IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application No. 10/701,022

Applicant: Anderson et al.

Filed: November 4, 2003

TC/AU: 1653

Examiner: Anne Marie Sabrina Wehbe

Docket No.: 219974 (Client Reference No. E-189-1989/3-US-04)

Customer No.: 45733

DECLARATION UNDER 37 C.F.R. § 1.132 OF DR. STEVEN A. ROSENBERG

1. I, Steven A. Rosenberg, am a co-inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. I created the TNF protocol which is described in the instant patent application as Example 4. Under my supervision, my laboratory carried out this protocol in at least one human patient.

3. As described in Example 4 of the instant patent application, the TNF protocol comprises transducing a mixed population of cells obtained from a patient by administering to the population of cells a retroviral vector encoding a gene, i.e., TNF, for expression of the gene. The TNF protocol is not specific for the transduction of T-lymphocytes. For instance, the retroviral vector used in the TNF protocol is not specific for gene expression in T-lymphocytes, e.g., it does not contain a T-lymphocyte-specific promoter, nor does the retroviral vector target a

T-lymphocyte-specific molecule for infection. Further, since the retroviral vectors are administered to a mixed population of cells, it is likely that cells other than the T-lymphocytes of the mixed population are transduced. Therefore, the protocol could be used for other cells, such as B-lymphocytes.

4. The TNF protocol has been the basis for several other gene therapy clinical trial protocols using different genes and different cells. For instance, this protocol has been the basis for the protocols used or being used in clinical trials for the following genes: IL-2, IL-15, IL-21, IFN-γ, and a T-cell receptor. Also, this protocol is the basis for the protocol currently used in a gene therapy clinical trial with $CD34^+$ stem cells. I believe that the protocol will work in these stem cells. The Internal Review Board (IRB) of the National Cancer Institute (NCI) also believes that this protocol will work with $CD34^+$ stem cells, since the IRB approved of this protocol before the clinical trial began and, to my knowledge, the IRB would not approve of a protocol unless there is sufficient reason to believe that it will work.

5. While I have not yet published the results of the TNF protocol, my laboratory has obtained data to support that the TNF protocol was successful in providing a human patient with a therapeutically effective amount of TNF. This patient has survived for more than 10 years due to the TNF gene therapy.

6. The TNF protocol was the basis for both of the protocol used in the gene therapy clinical trial of a T-cell receptor and the gene therapy clinical trial of IL-2. My laboratory has obtained data from both of these clinical trials indicating that therapeutically effective amounts

of the T-lymphocytes receptor or of IL-2 were provided to the humans in the clinical trials. One patient in the T-lymphocytes receptor gene therapy trial has survived for more than ten years due to the gene therapy.

7. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 8/15/05

Steven A. Rosenberg, M.D.

Interference Trial Section
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Paper No. 92

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

W. FRENCH ANDERSON,
R. MICHAEL BLAESE, and STEVEN A. ROSENBERG
(5,399,346),
Junior Party,

v.

JEFFREY R. MORGAN
and RICHARD C. MULLIGAN
(08/153,275),
Senior Party.

Interference No. 104,712

ERRATUM

In Paper No. 91, please make the following change:

Page 2, line 4, change "claims 1-14" to "claims 1-6, 8-11, 13, and 14". See Paper No. 88 at 56.

Sonja Despertt
SONJA DESPERTT
Trial Section Paralegal

cc (via facsimile):

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of	:	ANDERSON, ET AL.
Serial No.	:	904,662
Filed:	:	SEPTEMBER 8, 1992
For	:	GENE THERAPY
Group	:	1804
Examiner	:	JACQUELINE STONE

Honorable Commissioner of Patents
Washington, D.C. 20231

SIR:

W. French Anderson declares as follows:

1. He is one of the inventors of the referenced application.

2. He has attached hereto as Exhibit 1 a list of human gene therapy protocols, and to the best of his information and belief, the protocols listed as 1-38 have been approved by the Recombinant DNA Advisory Committee (RAC), a committee of the National Institutes of Health. All of such protocols (except for protocols No. 1-4, which are the protocols of the present application) were approved by the RAC after the above-named inventors had demonstrated the feasibility of human gene therapy through the ADA protocol of the present application.

3. To the best of his information and belief, RAC approval of the human gene therapy protocols 5-38 was obtained in a time period, which on the average was six months or less whereas it took over 3 years to obtain RAC approval for Anderson's initial gene therapy protocol (protocol 1, the ADA protocol of this application): April 24, 1987 (the date of the original pre-protocol submission to the Human Gene Therapy Subcommittee of the RAC)

to July 31, 1990. Now, most protocols are approved after an approximately eight week RAC review.

4. The approved protocols 5-38 are directed to human gene therapy with a variety of DNA sequences, employing a variety of delivery vehicles, and are directed to both ex vivo and in situ (in vivo) transduction of human cells. Thus, for example, such protocols include the following:

1. TNF, which is a secreted cytokine
2. IL-2, a secreted lymphokine;
3. LDL receptor, a membrane protein;
4. TK, an activatable viral gene;
5. HLA-B7, a cell surface antigen;
6. HIV-gp120, a surface antigen;
7. IL-4, a cytokine;
8. antisense-RAS, an antisense molecule to an oncogene;
9. p53, a tumor suppressor gene;
10. CF, an integral membrane transport protein;
11. GM-CSF, a hematopoietic colony-stimulating factor;
12. gamma interferon, a cytokine;
13. MDR, a membrane transport protein;
14. glucocerebrosidase, an intracellular enzyme;
15. mutated HIV, a viral protein;
16. Rev, a viral transcription factor;
17. anti-IGF-1, an antisense molecule to a cell growth factor; and
18. ribozyme, an RNA-cleaving RNA molecule.

In addition, the RAC-approved protocols encompass a wide variety of delivery means, such as retroviral vectors, adenovirus vectors, liposomes for delivery of plasmid DNA, and viral-producer cells.

In addition, such RAC-approved protocols encompass both ex-vivo transduction and in situ (in vivo) transduction of cells. Thus, for example, the TK protocol involves the use of a producer cell, which transduces cells in vivo.

The 5 CF protocols involve intratracheal or intranasal infusion of an adenovirus vector for transduction of cells in vivo.

In addition, such RAC-approved protocols include a protocol for direct injection into a cancer mass for transduction of cells in vivo.

5. To the best of his information and belief, the RAC does not approve a human gene therapy protocol unless there is a reasonable expectation of efficacy. In his opinion, the rapid approval of the human gene therapy protocols 5-38, in large part, resulted from the fact that the inventors of the present application had demonstrated the feasibility of human gene therapy through protocol 1, the ADA protocol of the present application. In particular, the demonstration of the feasibility of human gene therapy through the ADA protocol indicated that concerns, such as those raised by the Examiner on page 3 of the Office Action in the present application, with respect to the inappropriateness of human gene therapy, had been obviated, whereby it was now possible for those skilled in the art to design and obtain RAC approval for a wide variety of human gene therapy protocols.

6. Prior to the inventors demonstration of the feasibility of human gene therapy, there were no approved human gene therapy protocols. After such demonstration of the feasibility of human gene therapy, in a period of less than three years, there exists 37 additional approved human gene therapy protocols in the United States. In his opinion, the design and approval of such human gene therapy protocols was enabled by the inventors demonstration that human gene therapy is feasible.

7. He has attached hereto as Exhibit 2 a summary of the Institutions involved with human gene therapy protocols.

8. He has attached hereto as Exhibit 3 a graph which indicates the cumulative number of patients through the early part of 1993 who have received gene therapy. The first point on the therapy curve is the first patient on the ADA protocol of the present application. The graph of Exhibit 3 is incomplete in that it covers only to May 1, 1993. The graph illustrates the significant increase in human gene therapy patients, and the rapidity of such increase, after the inventors demonstrated the feasibility of human gene therapy.

9. He declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

W. French Anderson

W. FRENCH ANDERSON

Dated: October 15, 1993

EXHIBIT 1

Human Gene Therapy Clinical Protocols

1. ADA Deficiency - NIH
2. TNF/TIL/Melanoma - NIH
3. TNF/Cancer - NIH
4. IL-2/Cancer - NIH
5. Liver LDL Receptor/FH - Univ. of Michigan
6. TK/Ovarian Cancer - Univ. of Rochester
7. HLA-B7/Melanoma:1 - Univ. of Michigan
8. CTL/TK/AIDS - Univ. of Washington
9. IL-2/Neuroblastoma - St. Jude
10. TK Producer Cells/Brain Cancer - NIH

11. IL-2/Melanoma - Sloan-Kettering
12. IL-2/Renal Cell Cancer - Sloan Kettering
13. HIV gp120/AIDS:2 - Viagene
14. IL-4/Cancer - Univ. of Pittsburgh
15. Antisense Ras/p53/Lung Cancer - M.D. Anderson Hospital
16. CF/Lung/Adenovector - NIH
17. CF/Lung/Adenovector - Univ. of Michigan
18. CF/Nasal Epithelium/Adenovector - Univ. of Iowa
19. TK Producer Cells/Brain Cancer - Iowa Methodist
20. GM-CSF/Renal Cell Cancer - Johns Hopkins

21. CF/Lung/Adenovector - Univ. of Cincinnati
22. CF/Lung/Adenovector - Univ. of North Carolina
23. Gamma-Interferon/Melanoma - Duke University
24. MDR/Ovarian Cancer - M.D. Anderson Hospital
25. HLA-B7/Cancer:2 - Univ. of Michigan
26. Glucocerebrosidase/Gaucher - Univ. of Pittsburg
27. Glucocerebrosidase/Gaucher - NIH
28. HIV-IT(V)/AIDS - Univ. of Southern California
29. Rev-/AIDS - Univ. of Michigan
30. TK Producer Cells/Pediatric Brain Cancer - CHLA

31. MDR/Cancer - Columbia University
32. Anti-IGF-1/Cancer - Case Western Reserve
33. Il-2/Cancer - UCLA
34. MDR/Breast Cancer - NIH
35. Il-2/Melanoma - Univ. of Illinois
36. Il-2/Small Cell Lung Cancer - Univ. of Miami
37. TK Producer Cells/Pediatric Brain Tumor - St. Jude
38. Ribozyme/AIDS - UCSD

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39. HIV gp120/AIDS:1 - Viagen

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40. Factor IX/ Hemophilia B - Fudan Univ., Shanghai (China)
41. IL-2/Cancer - University Hospital, Leiden (Netherlands)
42. ADA Deficiency - San Raffaele Sci. Inst., Milan (Italy)
43. ADA Deficiency - TNO, (The Netherlands + France, England)

Date: 10/1/93

EXHIBIIT 2

SUMMARY

Human Gene Marker/Therapy Clinical Protocols

Number of Marker Protocols: 21
Number of Therapy Protocols: 43
Total Number of Protocols: 64

Institutions: 29

NIH	13	(5+8)
St. Jude	7	(5+2)
M.D. Anderson	5	(3+2)
Univ. of Michigan	5	(0+5)
Univ. of Washington	4	(3+1)
Univ. of Pittsburgh	3	(1+2)
Viagen	2	(0+2)
Sloan-Kettering	2	(0+2)
USC	2	(0+2)
UCLA	2	(1+1)

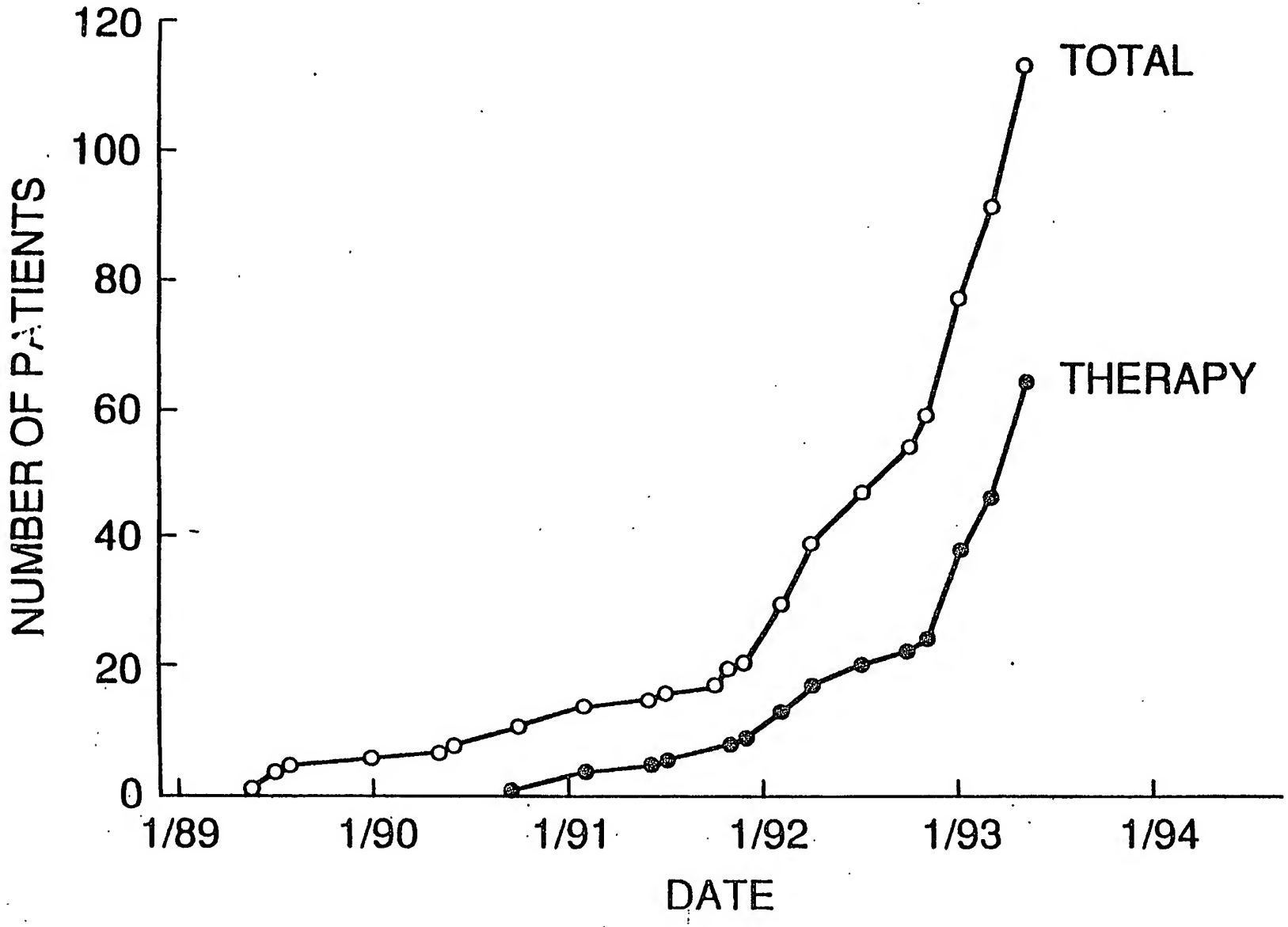
One each:

Marker:	Therapy:
CLB, Lyon	Fudan University, Shanghai
Indiana	University Hospital, Leiden
	SRM, Milan
---	TNO, The Netherlands (Eng, Fr)
Baylor	---
	Univ. of Rochester
	Univ. of Iowa
	Iowa Methodist
	Johns Hopkins
	Univ. of Cincinnati
	Univ. of North Carolina
	Duke University
	Columbia University
	Case Western Reserve
	UC San Diego
	Univ. of Miami
	Univ. of Illinois, Chicago

10/1/93

EXHIBIT 3

GENE MARKER/THERAPY CLINICAL PROTOCOLS: CUMULATIVE NUMBER OF PATIENTS



becco's minimum essential medium (DMEM) in the presence of polybrene (8 μg/ml) as described (*18*). Cells were washed twice in phosphate-buffered saline (PBS), resuspended in fresh medium, and cultured for 3 to 4 days. Transduced cells were tested for the presence of helper virus and cryopreserved until use.

36. BM mononuclear cells were obtained as a Ficoll fraction and grown for 2 to 3 days in complete DMEM at a density of 6×10^5 to 8×10^5 cells/cm^2 (*35*). T cell depletion and progenitor cell enrichment were obtained as described (*3, 35*). Gene transfer was carried out by multiple infection cycles with cell-free, helper virus–tested viral supernatants in the presence of polybrene (8 μg/ml) (*35*). BM cells were maintained in a long-term culture system over adherent layers without addition of exogenous growth factors, and infected during the first 3 days of culture. Transduced cells were tested for the presence of helper virus and cryopreserved until use. At that time, the transduced cells were washed, resuspended in normal saline containing 4% human albumin, and reinfused into the patient.
37. C. Bordignon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86, 6748 (1989).
38. PHA blasts or antigen-specific T cells were cloned by limiting dilution. The relative frequencies of transduced cells was obtained by comparing the precursor frequency in the absence and presence of G418 (800 μg/ml). G418-resistant T cell clones were isolated and maintained as described (*44, 45*).
39. The relative frequencies of transduced BM progenitor cells were obtained by comparing the frequency of CFU-G, CFU-GM, BFU-E, and CFU-GEMM cells in the absence and presence of increasing doses of G418 (0.7, 1.0, 1.5 mg/ml) as described (*37*). In selected experiments, individual G418-resistant colonies were collected for analysis of vector transduction and expression.
40. M. J. Barnett *et al.*, *Blood* 84, 724 (1994).
41. C. Bordignon *et al.*, data not shown.
42. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989).
43. P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* 1, 327 (1982).
44. A. Lanzavecchia, *Nature* 314, 537 (1985).
45. P. Panina-Bordignon *et al.*, *Eur. J. Immunol.* 19, 2237 (1989).
46. Y. Choi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86, 8941 (1989).
47. E. Y. Loh, J. F. Elliot, S. Cwirla, L. L. Lanier, M. M. Davis, *Science* 243, 217 (1989).
48. T. cell receptor V_β-chain usage was analyzed on transduced T cell lines by reverse transcriptase–PCR. Briefly, total RNA was reverse transcribed with oligo(dT) and oligo(dG) primers and subjected to PCR with V_β- or C_β-specific oligonucleotides (*46*) or to anchored PCR with a C_α-specific oligonucleotide as described (*47*). Amplified products were analyzed by agarose gel electrophoresis.
49. We are indebted to L. Ruggieri and A. Wack for performing some of the ex vivo and in vitro analyses of gene transfer frequency; to the nurses and clinical staff of the Clinica Pediatrica, School of Medicine, University of Brescia, for skilled and dedicated care; to A. Arrighini and A. Crescenzo for clinical assistance in the extended care of the two patients; to A. Plebani for dosing specific antibody production; to M. Hershfield, P. Dellabona, and A. Ballabio for helpful discussions; and to Enzon, Inc., and Ophan Europe for providing PEG-ADA before commercial distribution. Supported by grants from Telethon, the Italian National Research Council, and the Italian Ministry of Health (IV-VII AIDS Projects).

28 May 1995; accepted 27 September 1995

T Lymphocyte–Directed Gene Therapy for ADA$^-$ SCID: Initial Trial Results After 4 Years

R. Michael Blaese,* Kenneth W. Culver, A. Dusty Miller, Charles S. Carter, Thomas Fleisher, Mario Clerici,† Gene Shearer, Lauren Chang, Yawen Chiang, Paul Tolstoshev, Jay J. Greenblatt, Steven A. Rosenberg, Harvey Klein, Melvin Berger, Craig A. Mullen,‡ W. Jay Ramsey, Linda Muul, Richard A. Morgan, W. French Anderson§

In 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of two children with severe combined immunodeficiency (ADA$^-$ SCID). The number of blood T cells normalized as did many cellular and humoral immune responses. Gene treatment ended after 2 years, but integrated vector and ADA gene expression in T cells persisted. Although many components remain to be perfected, it is concluded here that gene therapy can be a safe and effective addition to treatment for some patients with this severe immunodeficiency disease.

The possibility of using gene transfer as a therapy for human disease has great appeal. The decision to enter clinical trials awaited the development of safe and efficient techniques of gene transfer and improved understanding of the basic pathology and biology underlying likely candidate diseases and target cells. The advent of useful retroviral vectors that permitted relatively high efficiency gene transfer and stable integration was a critical advance (*1, 2*), as was the demonstration that this procedure of gene transfer could be effectively and safely used in humans (*3*).

Severe combined immunodeficiency secondary to a genetic defect in the purine catabolic enzyme adenosine deaminase [ADA$^-$ SCID] is characterized by defective T and B cell function and recurrent infections, often involving opportunistic pathogens. Large amounts of deoxyadenosine, an ADA substrate, are present in these patients; deoxyadenosine is preferentially converted to the toxic compound deoxyadenosine triphosphate in T cells, disabling the immune system (*4*).

Because this disease is curable by allogeneic bone marrow transplantation given without pretransplantation cytoreductive conditioning, it was initially assumed that gene therapy should be directed at the bone marrow stem cell. However, initial attempts to use stem cell gene transfer in primates resulted in only low-level, transient gene expression, insufficient for clinical use. The observation that the only donor cells detected in some patients "cured" by allogeneic bone marrow transplantation was their T cells—the others remaining ADA-deficient (*5*)—raised the possibility that T cell–directed gene therapy also might be a useful treatment.

The introduction of enzyme replacement with ADA-containing erythrocytes (*6*) or with bovine ADA conjugated with polyethylene glycol (PEG-ADA) (*7*) has made this approach feasible. PEG-ADA has provided noncurative, life-saving treatment for ADA$^-$ SCID patients; with this treatment, most patients have experienced weight gain and decreased opportunistic infections. Full immune reconstitution has been less regularly achieved with enzyme therapy. T cell function as measured by in vitro mitogen responses improved in most patients, but fewer patients recovered consistent immune responses to specific antigens [for instance, as measured by normal delayed-type hypersensitivity (DTH) skin test reactivity] (*8–10*). Nearly all PEG-ADA–treated patients showed increased peripheral T cell counts, which provided a source of T cells for gene correction not available without enzyme therapy. Furthermore, enzyme treatment could be continued during the gene therapy trial so that the ethical dilemma of withholding or stopping a life-saving therapy to test an unknown treatment could be avoided.

The adenosine deaminase complementary DNA (cDNA) (*11*) is 1.5 kb and fits within a retroviral vector. With the use of an ADA-containing retroviral vector, ADA-deficient T cell lines were transduced to express normal amounts of ADA; this rendered them normally resistant to intoxication and growth inhibition when challenged with deoxyadenosine (*12, 13*). Next, studies in mice, rabbits, and nonhuman primates using T cells modified with retroviral vectors showed normal cell survival and function after their reintroduction into recipient animals (*14*). Finally, Bordignon and colleagues (*15*) showed that ADA gene–corrected T cells acquired a survival advantage compared with uncorrected ADA-deficient cells when transplanted into immunodeficient, but ADA-

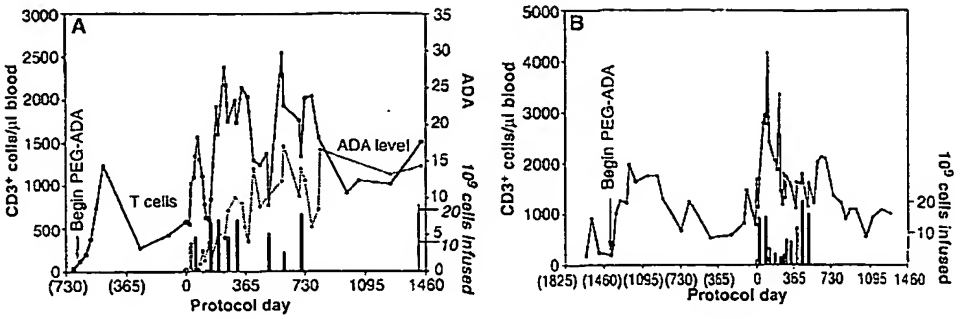


Fig. 1. Peripheral blood T cell counts since the time the diagnosis of ADA deficiency was made, dates of treatments, and the total number of cells infused for each patient. ADA level is measured in nanomoles of adenosine deaminated per minute per 10^8 cells. Vertical bars indicate the dates of cell infusion, and their height represents the total number of nonselected cells infused at each treatment. The T cell numbers represent total CD3-bearing T cells determined by standard flow cytometric analysis. (A) Patient 1 began gene therapy on 14 September 1990 (protocol day 0) and received a total of 11 infusions. Cellular ADA enzyme level is indicated by the dashed line. ADA activity was determined as described (*13*, *25*). Values shown are the mean of duplicate samples and represent EHNA-sensitive ADA enzyme activity. (B) Patient 2 began gene therapy on 31 January 1991 (protocol day 0) and received of a total of 12 infusions.

normal BNX recipient mice.

The clinical protocol used here has been described elsewhere (*16*). Patients with documented ADA$^-$ SCID were eligible if they did not have a human lymphocyte antigen–matched sibling as a potential donor for marrow transplantation and if they had been treated with PEG-ADA for at least 9 months without full immune reconstitution. T cells were obtained from their blood by apheresis, induced to proliferate in culture, transduced with the ADA retroviral vector LASN, culture-expanded, and then reinfused into the patient after 9 to 12 days (*17*). No selection procedure was used to enrich for gene-transduced cells.

The clinical histories and ADA gene mutations of each patient have been reported (*18*, *19*). Patient 1 presented with infection at 2 days of age and had recurrent infections and very poor growth until 26 months of age, when the diagnosis of ADA deficiency was established and she was started on PEG-ADA [30 U per kilogram of body weight per week (30 U/kg/week)]. Treatment with PEG-ADA enzyme for approximately 2 years had resulted in significant, but incomplete, benefit. With PEG-ADA she gained weight, had fewer infections, and transiently developed a normal peripheral blood T cell count (Fig. 1A), and her T cells had acquired the ability to respond to mitogens in vitro. However, significant immune deficiency persisted, including recurrence of her T lymphopenia (Fig. 1A), DTH skin test anergy (Table 1), depressed in vitro immune reactivity to specific antigens such as tetanus toxoid, failure to generate normal cytotoxic T cells to viral antigens or allogeneic cells, defective immunoglobulin production and absent or weak antibody responses to several vaccine antigens, and borderline isohemagglutinin titers (Table 1). At 4 years of age, she was enrolled in this trial.

The course of disease in patient 2 (who was 9 years old when enrolled in the trial) was milder than that seen in classic SCID (*19*). She had her first serious infection at age 3, and septic arthritis at age 5; the diagnosis was finally established at age 6 when significant lymphopenia with ADA deficiency was confirmed. This patient had an excellent initial improvement in peripheral T cell numbers after the start of PEG-ADA therapy (30 U/kg/week) at age 5, but lymphopenia recurred in the third and fourth years of enzyme treatment (Fig. 1B). During the year before gene therapy, repeated evaluation of her immune system showed persisting immunodeficiency, but less severe than that in patient 1. Despite 4 years of enzyme treatment, DTH skin test reactivity was absent (Table 1), cytotoxic T cells to viral antigens and allogeneic cells were deficient, and isohemagglutinins were barely detectable. However, illustrating the variability seen in the responses of patient 2 over time, blood lymphocytes that were cryopreserved from the day the clinical trial began and tested later showed normal cytotoxic activity to allogeneic cells.

Within 5 to 6 months of beginning gene therapy, the peripheral blood T cell counts for patient 1 (Fig. 1A) rapidly increased in number and stabilized in the normal range and have remained normal since that time (*20*). ADA enzyme activity, nearly undetectable in her blood lymphocytes initially, progressively increased in concentration during the first 2 years of treatment to reach a level roughly half the concentration found in heterozygous carriers (expressing only one intact ADA allele) and has re-

Table 1. DTH skin test reactivity and isohemagglutinin titers in sera of each patient at various times during the treatment protocol. Skin tests were applied as Multitest (Pasteur Merieux, Lyon, France) and scored according to the manufacturer's instructions 48 to 72 hours after being placed. Seven antigens were placed on the dates indicated, although only five were technically satisfactory on day 1252 for patient 1 and on day 1118 for patient 2. Isohemagglutinin titers were determined by standard blood bank techniques (*34*). Ninety five percent of normal children over the age of 2 years will have a titer of ≥1:16 and 82% will have a titer ≥1:32 (*35*). ND, not done. For the DTH skin tests, positive tests were elicited; T, tetanus toxoid; D, diphtheria toxoid; C, *Candida albicans*; P, Proteus antigen; S, streptococcal antigen; OT, old tuberculin.

Protocol day	Isohemagglutinins	DTH skin tests
	Patient 1	
−9	16	None (0/7)
115	256	ND
251	128	ND
314	32	T, D, C
455	32	T, D, C, S, P
510	64	ND
707	32	ND
1252	ND	D, C, P
	Patient 2	
−122	4	None (0/7)
−9	4	ND
90	256	ND
186	128	ND
291	128	ND
501	128	T, D, C, S, OT
676	64	ND
957	16	ND
1118	ND	T, D, S, P

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mained at that level since (Fig. 1A). Thus, both the reconstituted number of peripheral blood T cells and the elevated T cell ADA enzyme concentration have persisted since the patient's last treatment, indicating that peripheral T cells can have an unexpectedly long life-span and that gene expression from the retroviral vector has not been silenced over this period.

Patient 2, who had variable immune reactivity before enrollment, responded to the institution of lymphocyte infusions, with her peripheral T cell count rapidly increasing to levels in the high normal range (Fig 1B). Beginning with infusion 5, which included protocol modifications to partially deplete CD8 cells from the initially cultured cell population (*21*), her T cell count fell into the mid-normal range, where it persisted throughout the treatment period and for a year after the last cell infusion. In contrast to those in patient 1, ADA enzyme levels in the circulating T cells of patient 2 did not rise significantly above the small amounts seen before gene therapy treatment (~1.5 nmol/10^6 cells per minute).

The differences in final lymphocyte ADA concentration are consistent with the levels of gene transfer reached in these patients. For several months in the second protocol year during which cell infusions were not given, LASN vector sequences detected by polymerase chain reaction (PCR) maintained a stable frequency in the peripheral blood of patient 1 at a level greater than the PCR-positive control standard containing the equivalent of 0.3 vector copies/cell (Fig. 2). By contrast, although vector-containing cells were also stably detected throughout a similar period in patient 2, their level reached only a value equivalent to 0.1 to 1.0% of her circulating cells carrying the inserted ADA vector.

The principal contributor to the difference in the final frequency of LASN vector–modified T cells in patients 1 and 2 was the low gene transfer efficiency in the cells of patient 2; this was consistently only a tenth or less of what was routinely achieved in the cells from patient 1. Despite the gross differences in the final proportion of vector-containing cells reached in these two patients, both CD4 and CD8 T cell populations from each have remained consistently positive for integrated vector sequences since the first infusion through protocol day 1480 for patient 1 and through protocol day 1198 for patient 2 (Fig. 2).

To more accurately measure the proportion of vector-containing cells in patient 1, we performed quantitative Southern (DNA) hybridization analysis for vector sequence on DNA isolated from her peripheral blood T cells at different days during the course of this protocol. On protocol days 816 and 1252, which represent samples taken 109 and 545 days after the last treatment, the vector concentration was at the level of approximately one vector copy per cell (Fig. 3). Longitudinal studies of samples obtained throughout the study show that this large amount of integrated vector was reached by infusion 8 (D707) and that it has remained in this range since that time (*22*).

The use of a restriction endonuclease that cuts only once within the vector sequence does not give detectable bands (Fig. 3), indicating that the population of blood T cells at these dates is not oligoclonal with respect to integrated vector. Vector-derived mRNA was readily detected by reverse transcription (RT)–PCR at these same times (Fig. 3), confirming that vector expression persisted and was correlated with the presence of ADA enzyme activity in her circulating T cells.

To evaluate the effect of gene therapy on the immune function of these two patients in addition to its beneficial effect on T cell numbers, we performed a panel of immunologic studies both before, and at various times after, treatment. DTH skin test reactivity to common environmental and vaccine antigens tests the overall competence of the cellular immune system because a response depends on the full complement of cellular functions, not just cell proliferation or secretion of a single cytokine (Table 1). Patient 1 was anergic before our protocol treatment despite nearly 2 years of PEG-ADA treatment. Eight months after the initiation of gene therapy (protocol day 251), she had a brisk DTH response to a single intradermal skin test with tetanus toxoid. By protocol day 455, DTH responses to five of seven antigens were present, and this increased responsiveness has persisted, through day 1252.

Before the protocol, patient 2 had no positive DTH skin test (Table 1). At protocol day 501, five positive DTH skin tests were elicited, and this increased DTH reactivity had persisted when she was last tested on day 1118. She also acquired palpable lymph nodes and visible tonsils during the period of protocol treatment.

To corroborate the improved immune function indicated by these DTH tests, we evaluated the capacity of peripheral T cells from our patients to produce interleukin-2 (IL-2) or to kill antigenic target cells in vitro. In several patients treated with PEG-ADA, in vitro T cell proliferative responses to mitogens may normalize, whereas responses to specific antigens are less improved (*7–10*). During PEG-ADA treatment before gene therapy, T cells from patient 1 produced IL-2 in response to stimulation with

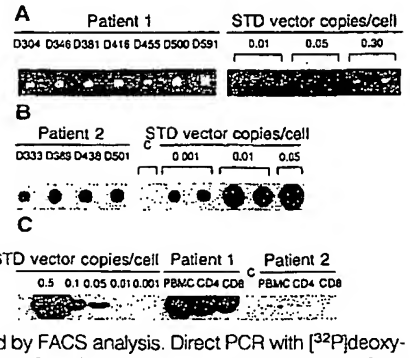

Fig. 2. PCR evaluation of the frequency of LASN vector–positive cells in the blood of patients 1 and 2 at various protocol days. (**A**) Cells from patient 1 for protocol days (D) 304 to 591 (see Fig. 1A). PCR analysis was performed as described (*26*) in an ethidium-stained gel. (**B**) Cells from patient 2 for protocol days (D) 333 to 501 (see Fig. 1B). PCR products were probed with ^{32}P-labeled *neo* gene as described (*26*). (**C**) Purified CD4$^+$ and CD8$^+$ cell subpopulations from patient 1 (D1480) and patient 2 (D1198) prepared by separation of peripheral blood mononuclear cells (PBMCs) by fluorescence-activated cell sorting (FACS). The purity of the separated T cell subpopulations from which DNA was extracted exceeded 98%, as confirmed by FACS analysis. Direct PCR with [^{32}P]deoxycytosine triphosphate was performed as described (*27*). Standards (STD) were prepared from DNA obtained from cell mixtures of a known proportion of LASN-transduced cells containing a single vector insert mixed with vector-negative cells. C, vector-negative control cells.

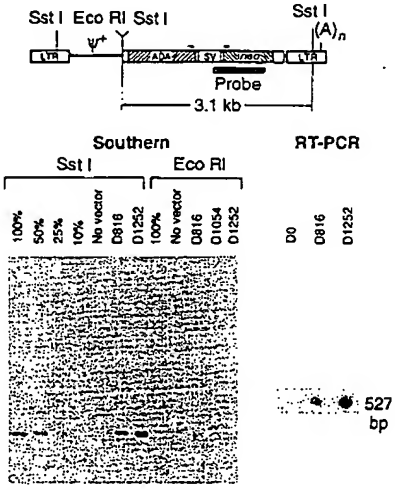

Fig. 3. Quantitative Southern hybridization analysis of DNA prepared from the blood mononuclear cells of patient 1 on protocol days (D) 816 and 1252 (*28*). DNA digested with Sst I should yield a single restriction fragment of 3.1 kb containing both the vector *neo* and ADA genes. Eco RI cuts only once within the vector sequence, and therefore a detectable band would indicate that a predominant clone with a single unique vector integration site was present in that blood sample. None was detected. Polyadenylated mRNA was extracted from the patient cells on days 0, 816, and 1252 and analyzed for vector message by RT-PCR (*29*). The primer locations used are indicated as short solid lines above the vector diagram. SV, SV40 early promoter; $(A)_n$, polyadenylation site; Ψ, extended retrovirus packaging signal. Hatched regions indicate protein coding regions.

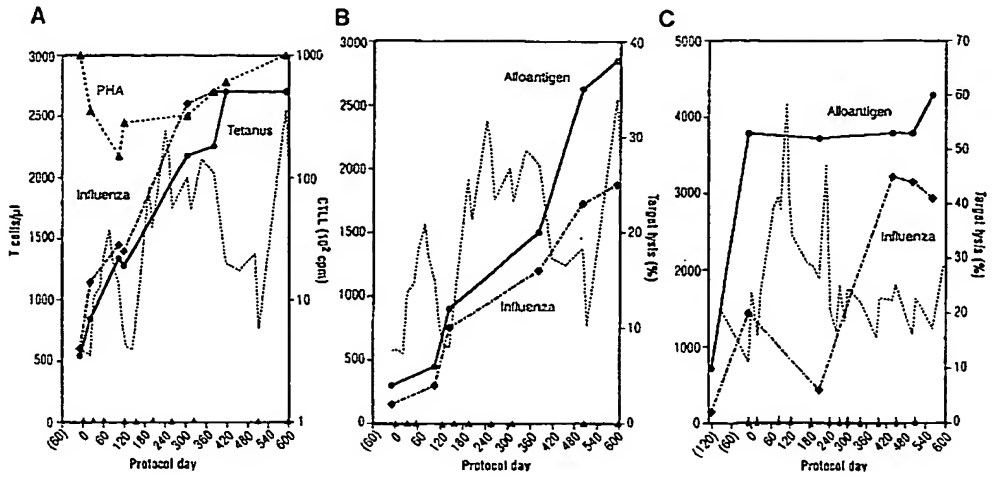

Fig. 4. Evaluation of the in vitro cellular immune responses of blood T cells from patients 1 and 2 at various times before and during the gene therapy trial. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (**A**) Production of IL-2 by cultured cells from patient 1 after stimulation with the mitogen PHA and with the specific antigens tetanus toxoid and influenza A virus as described (*30*). IL-2 was quantitated by bioassay measuring the proliferation of the IL-2–dependent T cell line CTLL at a 1:2 dilution of the lymphocyte culture supernatant. The fine dashed line indicates the patient's T cell count for reference. Solid triangles along the base line indicate the dates of cell infusion. (**B**) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell line and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 1 as described (*31*). Lysis (as percent specific isotope release during a 6-hour incubation of effector and target cells at a ratio of 60:1) was measured after in vitro pre-stimulation for 7 days. Solid triangles along the base line indicate the dates of cell infusion. (**C**) In vitro killing of a ^{51}Cr-labeled, influenza A–infected autologous B cell and a ^{51}Cr-labeled allogeneic target B cell line by blood T cells from patient 2 as described above.

the mitogen phytohemagglutinin (PHA) (Fig. 4A) but were unable to produce IL-2 in response to stimulation with influenza A virus or tetanus toxoid, despite repeated immunization with these antigens. Over the first months of gene therapy, IL-2 production improved and became normal after 1 year (Fig. 4A). Again before gene therapy, patient 1's T cells failed to show significant cytolytic reactivity against either allogeneic cells or influenza A–infected target cells. Almost mirroring the steady increase in IL-2 production, she acquired normal in vitro cytolytic T cell responses to these antigens, reaching normal values in her second year of treatment. (Fig. 4B).

The results of these cytolytic assays for patient 2 are shown in Fig. 4C. Tests done 120 days before the beginning of gene therapy also showed impaired responses. However, cells that were obtained at the time of the first gene therapy infusion, cryopreserved, and subsequently tested some months later showed a normal cytolytic response to allogeneic cells. After a year on gene therapy, cytolytic T cell activity against influenza also became normal.

To evaluate the effects of our treatment on humoral immune function in these patients, we measured antibody responses to several antigens. Despite their PEG-ADA treatment, both patients 1 and 2 had only low or borderline titers of isohemagglutinins on repeated testing before gene therapy. Each patient showed significant elevations in the levels of these antibodies within 90 to 115 days of beginning treatment with gene-modified cells (Table 1). Isohemagglutinins are antibodies that react with group A and B red blood cell antigens and occur spontaneously as a result of environmental exposure to cross-reacting antigens. Isohemagglutinin responses are, therefore, less dependent on the timing of previous immunizations than are responses to common vaccine antigens. After gene therapy, each patient also had improvement in antibody responses to vaccines to *Hemophilus influenzae* B (HIB) and tetanus toxoid (Fig. 5). With enzyme therapy alone, peripheral lymphocytes from each patient were unable to produce immunoglobulin M (IgM) in vitro after stimulation with pokeweed mitogen (PWM), but made robust responses after a year on the gene therapy protocol (Fig. 5A). Immunoglobulin production to PWM depends on T cells; these results further confirm the reconstitution of T cell function associated with gene therapy.

The effects of this treatment on the clinical well-being of these patients is more difficult to quantitate. Patient 1, who had been kept in relative isolation in her home for her first 4 years, was enrolled in public kindergarten after 1 year on the protocol and has missed no more school because of infectious disease than her classmates or siblings. She has grown normally in height and weight and is considered to be normal by her parents. Patient 2 was regularly attending public school while receiving PEG-ADA treatment alone and has continued to do well clinically. Chronic sinusitis and headaches, which had been a recurring problem for several years, cleared completely a few months after initiation of the protocol.

This trial of retroviral-mediated gene transfer shows that the survival of reinfused transduced peripheral blood T cells is prolonged in vivo; the erroneous assumption that T cells would not have such long-term survival was often cited as a potential problem with this treatment strategy. Patient 1 has had a normal total peripheral T cell count since the last cell infusion, and the proportion of her circulating T cells carrying vector DNA has remained stable over that period. Further, expression of the ADA transgene under the influence of the retroviral long terminal repeat (LTR) promoter has persisted for a long period in vivo without obvious extinction. There have been swings in the level of ADA enzyme in her peripheral lymphocytes throughout the period of observation, but the level of blood ADA enzyme activity at 4 years (protocol day 1480) is equivalent to that found immediately after the last cell infusion 2 years earlier (Fig. 1A). Although the data have not yet been completely analyzed, blood obtained after 5 years showed continuation of this trend with, again, a normal T lymphocyte count and an equivalent ADA level.

The mechanism by which our treatment aided immune reconstitution in patient 2 is less clear. The responses of patient 2 to some in vitro immunologic tests were variable before beginning our treatment protocol, ranging from little or no detectable response to nearly normal responses on the blood sample from the day gene therapy began. This patient produced a normal antibody response to immunization with bacteriophage φX174 about a year before beginning gene therapy (*8*). Although we have shown several examples of depressed cellular and humoral immune responses that strongly improved after gene therapy, this highly variable immune reactivity while patient 2 was on PEG-ADA therapy alone complicates interpretation of the contribution of our therapy. There was a temporal relation between initiation of gene therapy and a normalized peripheral T cell count,

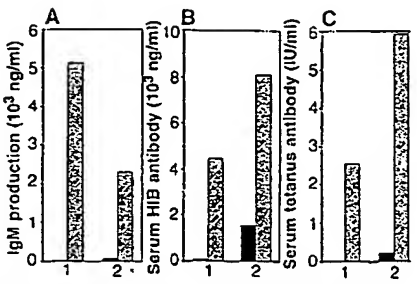

Fig. 5. Humoral immune function of patients 1 and 2 before (solid bars) and after (hatched bars) gene therapy. (**A**) IgM production by the patient's peripheral blood mononuclear cells in cultures stimulated with the T cell–dependent polyclonal activator PWM performed as described (*32*). "Before" samples were from D(−9). Follow-up cultures were at D500 (patient 1) and D560 (patient 2). In each case, the patient's cells stimulated with the T cell–independent B cell stimulant EBV (*33*) produced normal amounts of IgM (not shown), indicating intact B cell function before and after gene therapy, as expected. At least two normal subjects were included concurrently in each assay, and only those in which the controls responded appropriately are included here. (**B**) Serum antibody response to *Hemophilus influenzae* B. Patient 1 had failed to respond to two immunizations while on PEG-ADA alone [D(−9) shown]. Her response at protocol D591 is shown, after immunization. Patient 2 had some HIB-specific antibodies present before therapy [D(−122)], whose amounts increased without additional immunization during the protocol (D560). (**C**) Serum tetanus antibody. Patient 1 had negligible response to five separate tetanus immunizations before gene therapy [D(−48) shown] but responded briskly at D731, 24 days after re-immunization. Serum titers for patient 2 are shown for D(−9), 140 days after immunization while on PEG-ADA alone, and after receiving gene therapy (D592), 32 days after a booster tetanus immunization.

improved DTH, appearance of tonsils and palpable lymph nodes, normalized isohemagglutinin response, and improved PWM response, as well as other factors. In view of the relatively low level of ADA gene transfer achieved in this patient, the potential contribution of the infusions of the culture-activated T cells to the patient's response must also be considered. Perhaps ex vivo T cell activation somehow bypassed a differentiation block that PEG-ADA alone was unable to relieve. Despite the low final percentage gene transfer achieved, a 1% level of ADA gene–corrected cells could represent 10^9 to 10^{10} ADA-expressing T cells distributed throughout the body that could readily contribute to immune improvement.

Since the beginning of the trial, the dose of PEG-ADA enzyme given to each of our patients has been decreased by more than half (patient 1, 14 U/kg/week; patient 2, 10 U/kg/week), during which time their immune function has improved. By contrast, worsened immune function has been seen in other ADA$^-$ SCID patients when their dose of enzyme has been similarly reduced (*10*, *23*). We do not want to expose these patients to the potential risk of recurrent immunodeficiency by completely stopping PEG-ADA enzyme treatment until we have better information about the quality and duration of the immune improvement achieved by this first-generation gene therapy trial. The role of continued exogenous enzyme treatment will be clarified here or in companion studies attempting stem cell gene correction (*24*).

The safety of retroviral-mediated gene transfer has been a central concern. At least in the short and intermediate term, no problems have appeared in any clinical trial using these vectors. In the longer term, the theoretical potential for retroviral vectors to cause insertional mutagenesis remains the primary concern. To date, there has been no indication that malignancy associated with this process will be a complication of retroviral-mediated gene transfer.

Our trial here has demonstrated the potential efficacy of using gene-corrected autologous cells for treatment of children with ADA$^-$ SCID. Eleven children with this disease have been enrolled in various gene therapy protocols, each using different strategies and retroviral vector designs and focusing on different target cell populations. The experience gained from these approaches should provide guidance for gene therapy as a treatment for this disorder as well as for a larger array of inherited and acquired diseases.

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17. Peripheral T cells from the patients were collected by apheresis, isolated by density gradient centrifugation, washed extensively, and then cultured in 24-well culture plates in medium supplemented with 100 to 1000 IU/ml of recombinant IL-2 and 10 ng/ml of OKT3 to stimulate T cell proliferation. After 24 hours, half the medium was removed and replaced with supernatant containing the LASN retroviral vector supplemented with IL-2 and protamine (10 μg/ml) to give an initial multiplicity of infection of 1. The LASN vector contains the human ADA cDNA under the transcriptional control of the promoter-enhancer in the retroviral LTR and a neomycin phosphotransferase gene (*neo*) controlled by an internal SV40 promoter [R. A. Hock, A. D. Miller, W. R. A. Osborne, *Blood* 74, 876 (1989)] LASN was packaged with PA317 amphotropic retrovirus packaging cells (*2*). The LASN vector preparation, manufactured under good manufacturing practices by Genetic Therapy, Gaithersburg, MD, had a titer of 1×10^5 to 3×10^5. The cells were returned to the incubator and the transduction process repeated, with the addition of fresh retroviral supernatant and IL-2 twice daily for a total of three to five additions of vector. The cultured cells were transferred to gas-permeable culture bags at the conclusion of the transduction process. The proliferating T cell cultures were observed daily, split, and fed as necessary with periodic samples tested for viability and microbial contamination. Gene transfer efficiency was variable from treatment to treatment and patient to patient, ranging from 1 to 10% for patient 1 and 0.1 to 1% for patient 2. On days 9 to 12, the cultured cells were washed extensively with saline containing 0.5% human albumin and were then infused into the patient over a period of about 1 hour. During the 9 to 12 days of culture, the cell populations had expanded 17- to 135-fold. Preliminary studies testing the T cell receptor β gene repertoire showed that T cell cultures remained polyclonal for at least 3 weeks under these culture conditions. The culture period used in the clinical trial was held to half this time period to ensure a polyclonal T cell repertoire in the infused cell population.
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20. The protocol was reviewed and approved by the Clinical Research Subpanels of the NCI and NHLBI, the NCI Cancer Treatment and Evaluation Program (CTEP), the NIH Biosafety Committee, the Human Gene Therapy Subcommittee, the Recombinant DNA Advisory Committee, the Director of NIH, and the U.S. Food and Drug Administration. Informed consent was obtained from the parents of each patient.
21. Beginning with culture 9 for patient 1 and culture 5 for patient 2, the patients' lymphocyte populations obtained by apheresis were fractionated by adherence to flasks coated with CD8 monoclonal antibodies (Applied Immune Sciences) following the manufacturer's instructions. This protocol modification for CD8 depletion was introduced because both patients were developing a progressively inverted CD4-CD8 ratio. This effect was apparently the result of preferential growth of $CD8^+$ cells during the last 4 to 5 days of culture and the subsequent persistence of these infused $CD8^+$ cells in the circulation. Consequently, each subsequent apheresis sampled the recently increased number of $CD8^+$ cells, and thus the skewing of the ratio of CD4 to CD8 cells became compounded with each additional treatment. By partially depleting the apheresis sample of $CD8^+$ cells by an immunoaffinity selection process, the later treatments for each patient consisted of cells with a more balanced phenotype. The perturbation in normal CD4-CD8 cell proportions did not have detectable untoward effects for either patient.
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25. The ADA enzyme activity assay was performed in duplicate as described (*13*). Positive control cells were obtained from healthy normal donors and had a mean of 82 U (normal range, 66 to 102 U). Duplicate samples were run in the presence of the ADA enzyme inhibitor EHNA (30 μM). Specific ADA activity was calculated as total adenosine deaminating activity minus EHNA-resistant activity. EHNA-resistant activity represents metabolic activity of a nonspecific aminohydrolase present in human cells.
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28. Southern hybridization analysis for LASN vector consisted of the following: 10 μg of DNA was digested with Sst I and hybridized with a 728-bp Nco I fragment from LASN corresponding to the SV40 promoter and *neo* gene. DNA from K562-LASN cells served as a positive control.
29. RT-PCR analysis for LASN vector transcripts was as follows: 3 μg of polyadenylated RNA was treated with deoxyribonuclease and reverse-transcribed. The cDNA (0.3 μg) was amplified with LASN vector–specific primers in a 30-cycle PCR reaction. The oligonucleotides 5′-CAGCCTCTGCAGGGCAGAAC-3′ (corresponding to the 3′ end of the ADA gene in LASN) and 5′-GCCCAGTCATAGCCGAATAG-3′ (complementary to 5′ end of the *neo* gene in LASN) were used as primers. After electrophoresis and blotting, the sequences were hybridized with a 527-bp probe corresponding to the entire length of the predicted PCR product.
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36. The authors acknowledge superb technical and nursing support by C. Able, K. Snitzer, A. Roop, M. Brown, F. May, R. Gutieriez, M. Yu, H. Goetzman, C. Wannebo, B. Sink, and L. Top. In addition, we thank R. Sorensen for providing the initial patient blood samples, Applied Immune Sciences for CELLector T-150 flasks, and Genetic Therapy for clinical-grade LASN vector. Finally, we also thank our earlier collaborators D. B. Kohn, E. Gilboa, P. W. Kantoff, M. A. Eglitis, R. Moen, K. Cornetta, A. Gillio, R. J. O'Reilly, and C. Bordignon for their contributions that helped prepare the way for this clinical trial.

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Physical Map and Organization of *Arabidopsis thaliana* Chromosome 4

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A physical map of *Arabidopsis thaliana* chromosome 4 was constructed in yeast artificial chromosome clones and used to analyze the organization of the chromosome. Mapping of the nucleolar organizing region and the centromere integrated the physical and cytogenetic maps. Detailed comparison of physical with genetic distances showed that the frequency of recombination varied substantially, with relative hot and cold spots occurring along the whole chromosome. Eight repeated DNA sequence families were found in a complex arrangement across the centromeric region and nowhere else on the chromosome.

Arabidopsis thaliana has been adopted as a model organism for the analysis of complex plant processes by means of molecular genetic techniques (*1*). The increase in map-based cloning experiments makes the generation of a complete physical map of the *Arabidopsis* genome a high priority. In addition, the availability of such a map would enable the organization of the chromosome to be studied in more detail. Little is known about the organization of plant chromosomes, but the general picture is that of chromosomes carrying large numbers of dispersed [often retrotransposons (*2*)] and tandemly repeated DNA sequences (*3*). The relatively small (100 Mb) *Arabidopsis* genome has a much smaller number of repeated DNA sequences than do most other plant species; its five chromosomes contain ~10% highly repetitive and ~10% moderately repetitive DNA (*4*). The dispersion of most of these sequences among the low-copy DNA is unknown.

We discuss here a physical map, which we have presented on the World Wide Web (WWW) at URL: http://nasc.nott.ac.uk/JIC-contigs/JIC-contigs.html, of *Arabidopsis* chromosome 4, one of the two chromosomes carrying nucleolus organizing regions. The construction of this map allowed us to analyze the frequency of recombination along the whole chromosome, the integration of the physical with the cytogenetic map, the interspersion pattern of repeated and low-copy DNA sequences over the whole chromosome, and the arrangement of repeated DNA sequences over the centromeric region.

We generated the physical map by hybridizing probes to four yeast artificial chromosome (YAC) libraries (*5*), using colony hybridization experiments (*6*). The probes consisted of 112 markers genetically mapped to chromosome 4, 20 previously unmapped genes, random genomic fragments and sequences flanking transposable elements, and the 180–base pair (bp) repetitive element carried in pAL1 (*7*). Southern (DNA) blot analysis of YAC clones confirmed the colony hybridization results and revealed common restriction fragments in the different YAC clones hybridizing to a given marker. This demonstrated overlap between the inserts of the YAC clones. On the basis of these results, the YAC clones could be placed into 14 YAC contigs with a high degree of redundant YAC cover, ensuring an accurate map despite the presence of chimeric clones in the YAC libraries.

We generated YAC end fragments, using either inverse polymerase chain reaction (IPCR) or plasmid rescue (*8*), from YAC clones lying near the ends of each of the 14 contigs. The fragments were hybridized to Southern blots of YAC clones from adjacent contigs. In addition, YACs, as well as some of the end fragments generated by IPCR, were used to identify clones from a cosmid library of the Columbia ecotype (*9*). The cosmids were then used as new markers on the YAC libraries. These experiments reduced the number of contigs to four. In all but two instances, the end fragments revealed that the contigs were already overlapping. Experiments aimed at closing the last three gaps have been attempt-

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Molecular Analysis of T Lymphocyte-Directed Gene Therapy for Adenosine Deaminase Deficiency: Long-Term Expression *In Vivo* of Genes Introduced with a Retroviral Vector

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ABSTRACT

Peripheral blood lymphocytes from a patient with adenosine deaminase (ADA) deficiency were transduced *in vitro* with a replication-defective retroviral vector containing a human ADA-cDNA. Eighteen months after the last of a series of infusions of autologous retroviral vector-treated cells, vector sequences were detectable in DNA isolated from peripheral blood mononuclear cells (PBMCs), with an average copy number approaching one per cell. Increased ADA enzyme activity reaching approximately one-quarter normal levels was found in this population of cells. Other evidence of long-term retroviral vector expression *in vivo* included neomycin phosphotransferase (NPT) activity and demonstration of persistent vector mRNA by reverse transcriptase polymerase chain reaction (RT-PCR). No evidence of spontaneous reversion of either mutant endogenous ADA allele was found.

OVERVIEW SUMMARY

Children with severe combined immunodeficiency due to adenosine deaminase (ADA) deficiency have been treated with autologous T lymphocytes transduced *in vitro* with a retroviral vector containing both a normal human cDNA for ADA and a cDNA for neomycin phosprotransferase (neo). This report by Mullen *et al.* analyzes long-term expression of the retroviral vector genes in the first patient over a three and one-half year period of treatment and observation.

INTRODUCTION

Isolation and characterization of genes responsible for inherited diseases and development of gene transfer technology have led to the initiation of clinical trials of gene therapy for human disease. A prerequisite for successful gene therapy of many human diseases is stable, long-term expression *in vivo* of an exogenous gene in a physiologically relevant quantity. Critical to such an outcome are factors such as the lifespan of the transduced target cell *in vivo* and the continued expression of the introduced gene *in vivo*.

Adenosine deaminase (ADA) deficiency is an autosomal recessive disorder that accounts for roughly 20% of the cases of severe combined immunodeficiency (Hirschhorn, 1990). Lack of ADA enzyme activity leads to the accumulation of adenosine, deoxyadenosine, and their metabolites, which are preferentially toxic to lymphocytes. The ADA gene was cloned in the early 1980s (Daddona *et al.*, 1984; Valerio *et al.*, 1985; Wiginton *et al.*, 1984), and preclinical gene transfer studies using this gene have been carried out by a number of groups (Williams *et al.*, 1986; Kantoff *et al.*, 1987; Palmer *et al.*, 1987; Cournoyer *et al.*, 1991; van Beusechem *et al.*, 1992; Moritz *et al.*, 1993). One approach to treatment of this disorder is *ex vivo* retroviral vector transfer of a functional ADA gene into T lymphocytes, expansion of these cells *in vitro*, and reinfusion of these autologous genetically modified cells (Kantoff *et al.*, 1986; Culver *et al.*, 1990; Ferrari *et al.*, 1991; Braakman *et al.*, 1992). Two ADA-deficient patients were treated in this manner. Patient 1 exhibited significant lymphocyte transduction and an unequivocal increase in ADA activity. Patient 2 had less than

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1% vector transduced lymphocytes and no significant increase in ADA activity (Blaese *et al.*, 1995). Despite this, they exhibited similar increases in immunological function. Their clinical courses and the relative contributions of retroviral ADA gene transduction and infusion of activated lymphocytes expanded *in vitro* are described elsewhere (Blaese *et al.*, 1995). This report describes the long-term expression of an ADA gene in Patient 1 who received 11 such infusions over 23 months and who was followed without further intervention for an additional year and a half (Blaese *et al.*, 1990). We wished to learn how long lymphocytes transduced with retroviral vectors would persist *in vivo* and how well the genes encoded by such a vector would be expressed over time *in vivo*. Furthermore, we sought to demonstrate that the ADA found in the patient's T cells represented an authentic vector-derived gene product rather than enzyme production from an endogenous ADA gene (Brown *et al.*, 1994).

MATERIALS AND METHODS

Transduction of patient lymphocytes

The clinical protocol is described in detail elsewhere (Blaese *et al.*, 1990). Informed consent was obtained from the parents of each patient on the protocol. Each treatment consisted of infusion of approximately $1–2 \times 10^{10}$ autologous lymphocytes that had been exposed to LASN retroviral vector *in vitro* and cultured for a total of 10–11 days. Transduction efficiencies were usually in the range of 1–10% (Blaese *et al.*, 1995). The LASN vector is a replication-incompetent retroviral vector whose general structure is: [MoMLV LTR–human ADA cDNA–SV40 early region promoter–neomycin phosphotransferase gene] (Hock *et al.*, 1989). Clinical-grade vector was produced in PA317 amphotropic packaging cells (Miller *et al.*, 1986) (Genetic Therapy, Inc., Gaithersburg, MD).

ADA assay

ADA enzyme activity assay was performed as previously described (Kohn *et al.*, 1989). Peripheral blood mononuclear cells (PBMCs) were purified on a Ficoll gradient and washed in phosphate-buffered saline (PBS). Tissue culture cells were harvested and washed in PBS. Cells were centrifuged at $\sim 1,500 \times g$ in microfuge tubes and these pellets were stored at −70°C until assayed. Cells were resuspended at a concentration of 5×10^6 cells/ml in 100 m*M* Tris pH 7.4 and 1 mg/ml bovine serum albumin (BSA) and lysed by five rapid freeze–thaw cycles. Lysates were microfuged at $\sim 15,000 \times g$ for 2 min. A 10-μl amount of sample lysate containing the equivalent of 50,000 cells was incubated with 10 μl of 0.167 m*M* [^{14}C]adenosine (50 μCi/ml)(Sigma, St. Louis, MO) for 15 min. (The final concentration of adenosine in the reaction was 0.083 m*M*.) The enzyme reaction was terminated by addition of 20 μl of absolute ethanol and heating to 95°C for 5 min. Next, 50% of the sample was applied to a cellulose TLC sheet along with 3 μl of a nonradioactive solution of 10 m*M* deoxyadenosine, hypoxanthine, and deoxyinosine markers (Sigma, St. Louis, MO). The developing buffer was an aqueous solution with Na_2PHO_4 0.06 *M* pH 6.8, *n*-propyl alcohol 1.2%, and saturated ammonium sulfate 26% (vol/vol). After development and drying, the deoxyadenosine, hypoxanthine, and deoxyinosine spots were identified under shortwave UV light and cut from the sheet. Hypoxanthine and deoxyinosine (the deoxyadenosine metabolic products) were counted together while deoxyadenosine was counted separately in a liquid scintillation counter. Activity is expressed as nanomoles of adenosine deaminated/min per 10^8 cells. Duplicate samples were run in the presence of the ADA enzyme inhibitor EHNA (30 μM) (gift of R. Agbaria and D.G. Johns, Laboratory of Medicinal Chemistry, National Cancer Institute). Specific ADA activity was calculated as total adenosine deaminating activity minus EHNA-resistant activity. EHNA-resistant activity represents metabolic activity of a nonspecific aminohydrolase present in human cells (Schrader *et al.*, 1978; Daddona *et al.*, 1981; Ratech and Hirschhorn, 1981). Positive control cells were obtained from healthy normal donors and yielded on average 72 units (normal range 66–102 units).

Neomycin phosphotransferase (NPT) assay

This was performed as previously described (Reiss *et al.*, 1984) and represents ability of lysates of 5×10^6 cells to phosphorylate a neomycin analog *in vitro*. The strongest signal in patient samples was similar to the signal generated by a 1:100 to 1:1,000 dilution of K562-LASN cells, a strong positive control. The limit of detection is approximately 1 ng of purified NPT enzyme.

Detection of vector DNA

Southern Blots: A total of 10 μg of DNA was digested with *Sst* I and hybridized with a 728-bp *Nco* I fragment from LASN corresponding to the SV40 promoter and *neo* gene. DNA from K562-LASN cells served as positive control.

PCR for ADA Vector: A 10-μl amount of cell lysate (the equivalent of 5×10^4 cells) was used as template for a 30-cycle PCR reaction. The oligonucleotides 5′-CAGCCTCTGCAGGGCAGAAC-3′ (corresponding to the 3′ end of the ADA gene in LASN) and 5′-GCCCAGTCATAGCCGAATAG-3′ (complementary to 5′ end of the neomycin phosphotransferase gene in LASN) were used as primers. ^{32}P-labeled dCTP was present in the reaction. Products were run on a 6% polyacrylamide gel that was dried and used to expose film.

RT-PCR analysis for LASN vector transcripts

A 3-μg amount of poly(A)RNA was treated with DNase and reverse transcribed. Then 0.3 μg of cDNA was amplified with LASN vector-specific primers (5′-CAGCCTCTGCAGGGCAGAAC-3′ corresponding to the 3′ end of the ADA gene in LASN and 5′-GCCCAGTCATAGCCGAATAG-3′ complementary to the 5′ end of the neomycin phosphotransferase gene in LASN. Following electrophoresis and blotting, the sequences were hybridized with a 527-bp probe corresponding to the entire length of the predicted PCR product.

RT-PCR analysis of endogenous (nonvector) ADA-mRNA

A 0.3-μg amount of cDNA prepared from DNase-treated poly(A)RNA was amplified with primers corresponding to exon 5 of normal ADA (found in both endogenous and vector ADA)

and antisense to a distal untranslated portion of exon 12 (found only in endogenous ADA). The upstream primer was from exon 5 (~ bp 376 relative to the start codon) and consisted of the sequence: 5'-CCAGACGAGGTGGTGGC-3'. The downstream primer was 5'-GACTATTGAGATCATGGTCTTCTT-3' and corresponded to a normally untranslated region of exon 12 (~ bp 1,175) not present in the vector cDNA. Each PCR product was split into three aliquots and digested with no enzyme (−), *Bst* XI (X), or *Bgl* II (G). Following electrophoresis and blotting of the digest products, the filter was hybridized with a 345-bp *Bam* HI–*Bg* III fragment from LASN corresponding to exons 9–12 of ADA.

Cloning of lymphocytes

Clones were derived from PBMCs on day 985, 9 months after the final treatment. They were isolated by limiting dilution (Nutman, 1991) and expanded in tissue culture. Peripheral blood was fractionated on a gradient of Ficoll-Hypaque. PBMCs were activated with OKT3 (10 ng/ml) and interleukin-2 (IL-2) (100 IU/ml) and initially grown on 1.5×10^4 2,500 cGy irradiated allogeneic PBMCs at 0.1–10 patient cells per well in RPMI with 10% FCS in round-bottomed 96-well plates. Colonies emerging from plates with fewer than 32 positive wells were considered clonal. Culture time averaged 2 months and approximately 12×10^6 cells for each clone were generated.

Cell lines

TJF-2 is a human ADA-deficient T cell line immortalized with human T lymphotrophic virus type 1 (HTLV-1) (Kohn *et al.*, 1989). K562 is a human erythroleukemia cell line with ADA activity similar to that of normal human PBMCs (Table 1). These cell lines were transduced *in vitro* with LASN retroviral vector and selected in G418 1 mg/ml. The cultured pretreatment patient lymphocyte line represented Ficoll-Hypaque-purified venipuncture blood obtained before the first infusion of vector-treated cells. These PBMCs were stimulated with PHA, grown 4 weeks *in vitro*, and cryopreserved for 4 years.

RESULTS

Transduction and sampling of lymphocytes

Peripheral T lymphocytes from a previously described patient with ADA deficiency (Hershfield *et al.*, 1993) were transduced with the LASN retroviral vector containing a human ADA cDNA (Hock *et al.*, 1989). *In vitro* transduction effi-

TABLE 1. ADA ENZYME ACTIVITY IN UNCULTURED PBMCS AND IN LONG-TERM LYMPHOCYTE CELL LINES

Cells		*Vector DNA*[a]	*Vector RNA*[b]	*Percent normal ADA activity*[c]
Uncultured PBMCs	Normal donor	−	−	100
	Patient pretreatment	−	−	1
	Patient day 115	+	−	3
	Patient day 500	+	+	11
	Patient day 1252	+	+	18
Cell lines	Patient clone 1.1	+	NT[d]	61
	Patient clone 1.3	+	+	60
	Patient clone 1.7	+	+	141
	Patient clone 1.14	+	+	74
	Patient clone 1.20	+	NT	43
	Patient clone 1.21	−	−	24
	Cultured patient Pretreatment	−	−	10
	TJF-2	−	−	1
	TJF-2-LASN	+	+	407
	K562	−	−	98
	K562-LASN	+	+	3,387

Uncultured PBMCs were obtained from Ficoll-Hypaque-treated venipuncture blood. Patient clones were obtained from patient on day 985, 9 months after the final infusion of treated cells. Cultured patient pretreatment cells were PBMCs obtained prior to the first treatment that were stimulated with PHA *in vitro* and grown *in vitro* for greater than 7 weeks in tissue culture before analysis.

[a]Vector DNA +, Cells were positive for LASN vector DNA by Southern hybridization analysis of genomic DNA and by PCR using primers specific for vector.

[b]Vector RNA +, DNase-treated mRNA was RT-PCR positive for LASN vector using primers that were specific for LASN and that did not amplify cellular ADA mRNA.

[c]% normal ADA activity. EHNA-sensitive adenosine deaminating activity of cells as a percentage of normal peripheral blood PBMC activity. % normal ADA activity values for clones represent total adenosine deaminating activity; the limited number of cloned cells precluded repetition of the assay with the use of EHNA as a specific inhibitor of ADA.

[d]NT, Not tested due to lack of a sufficient number of cloned cells for RNA preparation.

ciencies were 1–10% as assessed by PCR on cell product samples obtained prior to infusion (Blaese *et al.*, 1995). Three infusions (#3 on protocol day 60, #8 on day 314, and #11 on day 707), each followed by a significant interval before subsequent infusions resumed, were studied in detail with respect to the presence of vector DNA, vector message measurable by RT-PCR, and vector product measured as ADA and neomycin phosphotransferase-2 (NPT) enzyme activity. For each of these cycles, PBMCs were sampled immediately prior to and at various times after cell infusions. These treatment-free evaluation periods were 55, 186, and 545 days, respectively.

Detection of retroviral vector DNA in patient lymphocytes

Vector sequences were readily detectable on Southern blots of this patient's PBMCs up to 545 days after the previous treatment. Both Southern blots (Fig. 1) and semiquantitative PCR (data not shown) indicted that the average copy number of vector sequences per cell increased progressively during the course of the treatment protocol and approached approximately one in the samples taken after the eleventh infusion cycle. The vector signal did not wane during the 186 days following infusion 8, protocol days 314–500, or in the 545 days following infusion 11, protocol days 707–1,252. Southern blots in which patient PBMC DNA was digested with *Eco* RI (which cuts only once in LASN) gave no evidence of a monoclonal or oligoclonal population of vector-containing cells (Blaese *et al.*, 1995).

ADA activity

ADA enzyme activity also increased progressively in the population of post-treatment PMBCs, approaching one-quarter normal levels in the period following the final infusion of ADA vector-modified cells (Fig. 1). This level of ADA activity is clinically significant. The increase in ADA enzyme activity in the post treatment PBMC samples suggested that the transduced LASN vector was its source. However, the ADA enzyme assay cannot distinguish between enzyme produced by the retroviral vector and endogenous ADA genes. Expression from the LASN vector was further tested in two ways: first, by measurement of NPT activity in the PBMCs and second, by detection of vector mRNA.

Expression of vector neo *gene*

The LASN vector contains two transgenes: human ADA driven by the promoter/enhancer in the MoMLV retroviral LTR

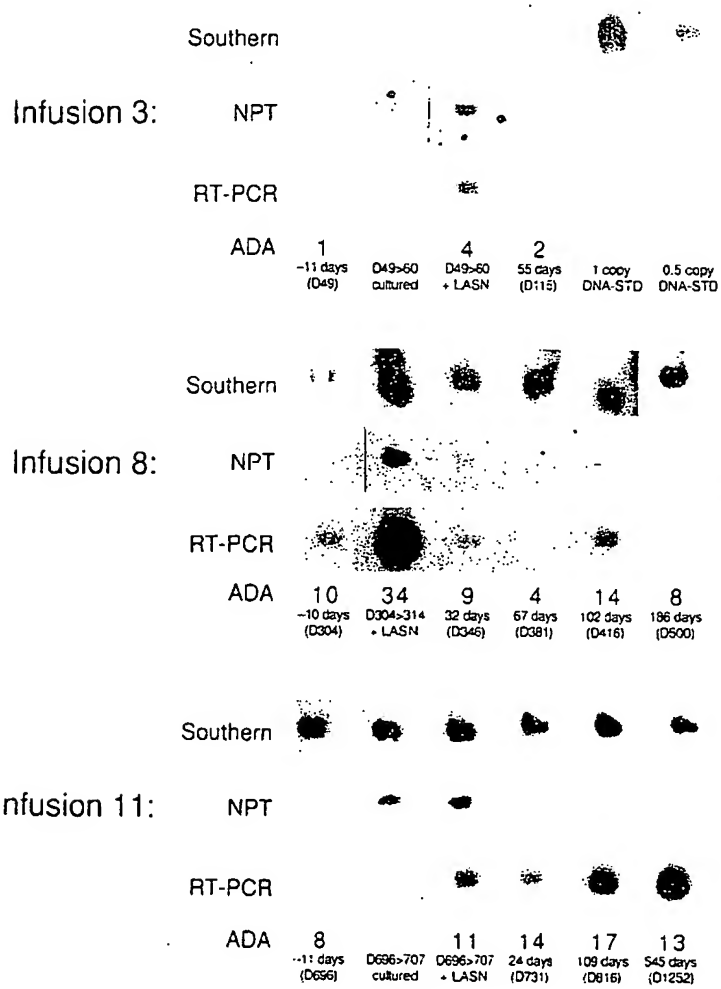

FIG. 1. LASN vector expression in patient PBMCs. Patient PBMC samples from infusion cycles 3, 8, and 11 were studied. The study date is indicated under each column. The number of days from the initiation of the study is indicated by "D" (*e.g.*, D115). The number of days relative to the infusion date for the infusion cycle is also indicated (*e.g.*, "55 days" relative to infusion 3). PBMCs from blood were directly assayed and are identified with a "D" number in parentheses, *e.g.*, (D115). Cells grown in culture without additional vector are designated "cultured." Those designated "LASN" were exposed to additional vector and were aliquots of the cells infused into the patient in the infusion cycle. For each point in time, a Southern blot was performed to identify the LASN vector. DNA from K562-LASN cells provided the positive control. NPT is neomycin phosphotransferase activity in lysates of 5×10^6 cells. Positive patient samples are similar in intensity to samples of 5×10^3 K562-LASN cells. RT-PCR represents LASN vector message in DNase-treated RNA. Positive patient signals from 0.3 μg of cDNA were similar in intensity to 0.3 ng of cDNA from K562-LASN. EHNA-sensitive ADA activity is described for each PBMC sample.

and a neomycin-resistance (*neo*) gene expressed by an internal SV40 early region promoter. *neo* gene activity is not normally present in human cells and thus the presence of its product, NPT, in cells would demonstrate transcription and translation of integrated vector sequences. PBMCs from the same days that were analyzed for vector presence by Southern blots were assayed for NPT activity (Fig. 1). NPT could be directly detected in samples from the patient's circulating T cells on several occasions during the course of our observations, confirming vector derived gene function.

Expression of vector ADA gene

Demonstration of NPT enzyme activity in the patient's T cells provides unambiguous evidence of vector expression. However, the *neo* and ADA genes are in separate transcriptional units and it could be argued that the ADA activity still could come from the patient's endogenous ADA genes. Conventional Northern blots were insensitive to the levels of vector ADA-mRNA in the PBMCs, compatible with the relatively modest amount of ADA and NPT activity in these cells. RT-PCR, however, detected vector ADA message in all the post-treatment PBMC samples with the exception of days 115 and 381 (Fig. 1).

Comparison of LASN vector expression in patient lymphocytes and in transformed cell lines

Increased ADA activity, NPT activity, and vector mRNA were found in patient PBMCs after treatment and the relative levels of these correlated with each other. In these studies, transformed human cell lines K562 and TJF-2 transduced with the LASN vector and selected in G418 were used as positive controls. In these cell lines, the vector-induced increases in ADA activity (Table 1) were much greater than in the patient's lymphocytes. Significantly greater NPT activity and vector mRNA were also seen in these cell lines (data not shown).

Analysis of endogenous ADA activity

As part of this post-treatment analysis, an attempt was made to clone patient lymphocytes and analyze the magnitude of vector expression in clonal populations. T cell clones were prepared by limiting dilution, without G418 selection, from the patient's peripheral blood drawn on protocol day 985. The patient's cells did not clone efficiently, but six clones yielded enough material before reaching senescence for characterization by Southern, RT-PCR, and ADA enzyme analyses. A summary of the analysis is contained in Table 1. Five of the six clones contained integrated LASN vector DNA. Each of the vector-positive clones exhibited significant ADA enzyme activity, ranging from 43% to 141% of that in normal PBMC (Table 1). Vector mRNA was detected in each of the three vector-positive clones analyzed. Clone 1.21 was negative for vector DNA by both Southern blot and PCR, and also negative for vector mRNA by RT-PCR. Surprisingly, clone 1.21 had 24% normal adenosine deaminating activity.

It is possible that the process of cloning may have selected for cells that had higher levels of adenosine deaminating activity. Consistent with this was the finding that a cell line derived from nontransduced, pretreatment PBMCs from this patient also contained some adenosine deaminating activity (Table 1). This cell line had been stimulated with phytohemoagglutinin (PHA) and IL-2, grown for 4 weeks, cryopreserved for 4 years, thawed, and grown again for 3 weeks. PBMCs obtained by venipuncture from the patient prior to therapy and not cultured had never shown any significant ADA activity. We and others have observed that upon occasion T cells from ADA(−)SCID patients will exhibit increased ADA enzyme activity upon *in vitro* activation and culture (Arredondo-Vega *et al.*, 1990). This finding raised the possibility that the increased ADA activity in post-treatment patient PBMCs might represent recovery of expression of an endogenous ADA gene or amplification of another enzyme that could deaminate adenosine.

In normal cells adenosine deaminating activity is largely due to ADA enzyme, whereas a nonspecific aminohydrolase contributes approximately 1% of the activity. These two enzymes can be distinguished by use of the enzyme inhibitor EHNA (Schrader *et al.*, 1978; Daddona *et al.*, 1981; Ratech and Hirschhorn, 1981). ADA is inhibited by EHNA whereas the nonspecific aminohydrolase is not. (All ADA values in Fig. 1 are EHNA sensitive.) In theory cells overexpressing either enzyme would have a survival advantage as they were activated and expanded *in vitro* for 10 days as part of the treatment protocol. To investigate this possibility, cultured pretreatment cells that had shown ADA activity were reassayed with the use of EHNA as a specific inhibitor of ADA enzyme (Table 1). It was found that half of the adenosine deaminating activity in these cultured cells was EHNA resistant and therefore represented amplified activity of the nonspecific aminohydrolase. Nonetheless, some authentic, EHNA sensitive ADA enzyme activity was also present in these cultured cells.

This raised the possibility that in the course of treatment a spontaneous reversion of the mutations in the patient's cells may have occurred. The mutations for this patient have been described (Santisteban *et al.*, 1993). One mutant allele (A) contains a Gly216 > Arg point mutation in exon 7 that creates a new *Bst* XI restriction site. A patient homozygous for this point mutation had less than 1% normal ADA activity and a severe clinical immunodeficiency (Hirschhorn *et al.*, 1991). The other allele (B) is a splice site mutation in intron 5 (a T + 6 > A transversion) that eliminates exon 5 from mRNA, resulting in a mRNA reading frame shift and a premature stop signal at codon 131 (in the middle portion of the protein).

Given the nature of the splice site mutation, it could be speculated that correct splicing might occur in culture-activated lymphocytes and be responsible for producing normal ADA protein. To evaluate this possibility, PBMC samples from five time points were subjected to RT-PCR using primers from exon 5 and a distal untranslated portion of exon 12. This primer set will not amplify vector sequences because the vector lacks this untranslated portion of ADA exon 12 and will not amplify improperly spliced mRNA from the endogenous allele B because such splicing eliminates exon 5. If proper splicing were occurring, two species of RT-PCR products should be seen in the PBMCs: one possessing the extra *Bst* XI site (from allele A with the point mutation) and one lacking this site (allele B). Following *Bst* XI digestion, mutant A yields a 485-bp product whereas the wild-type allele would yield a 596-bp product. Figure 2 demonstrates that in none of the samples analyzed was there evidence of properly spliced mRNA lacking the point mutation as judged by the lack of the 596-bp *Bst* XI-digested RT-

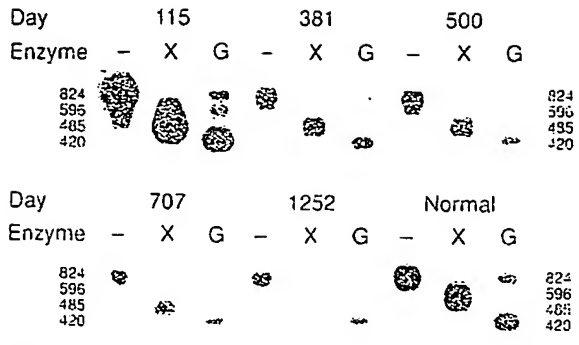

FIG. 2. RT-PCR analysis of endogenous (nonvector) ADA-mRNA. A 0.3-μg amount of cDNA was amplified with PCR using vectors that were specific for endogenous ADA and that fail to amplify LASN vector-derived mRNA. Each PCR product was split into three aliquots and digested with: no enzyme (–), *Bst* XI (X) or *Bgl* II (G). Normal and mutant ADA genes should both produce an 824-bp band that reduces to 420 bp with *Bgl* II digestion. (The larger bands in the "G" lanes represent incomplete digestion with *Bgl* II.) *Bst* XI digestion of the PCR product from a normal gene yields a 596-bp band, whereas the mutant ADA gene which contains an additional *Bst* XI site yields a 485-bp band. Samples are from the patient on the study day indicated or from a normal human donor. The bands corresponding to days 115, 381, and 500 represent an overnight exposure of the blot whereas the bands from days 707, 1,252, and the normal donor are from a 3-hr exposure of the same blot.

PCR product. Thus, it is very unlikely that the increased ADA activity *in vivo* came from reversion of either the point mutation in the one gene or proper splicing of the allele with the splice site mutation. The analysis does not exclude the possibility that some post-translational modification of the Gly216 > Arg mutant enzyme contributes to the increased ADA activity in the vector-negative cultured T cells, but no precedent for such a mechanism has been reported.

DISCUSSION

This molecular analysis of the extent and duration of expression of transgenes introduced into human peripheral T cells with retroviral vectors has demonstrated that prolonged transgene expression *in vivo* is possible with the current generation of retroviral vectors when used in primary T cells. It has been observed in some experimental systems that in nontransformed cells retroviral vector genes expressed *in vitro* may cease to be expressed when the vector-modified cells are returned to the living host (Palmer *et al.*, 1991). This experimental observation, as well as the observation that some vector-negative lymphocyte lines from this ADA-deficient patient exhibited some ADA activity, made it important to evaluate expression of the retroviral ADA vector critically.

The demonstration of NPT activity and vector mRNA in the patient's lymphocytes provides positive evidence for long-term expression. The failure to identify any reversions from the patient's mutant ADA genes also strengthens the conclusion that the increased ADA activity in the patient's lymphocytes was due to successful transfer and expression of the vector. In these studies we observed that the magnitude of expression in patient lymphocytes was only 0.1–1% of that seen in vector-treated control K562 or TJF-2 transformed cell lines. This difference may be due to different selection pressures. The transformed cell lines were selected *in vitro* with G418 whereas the patient's lymphocytes were not. It is highly likely that *in vivo* there was selective pressure for lymphocytes with greater ADA activity. Transduction efficiencies were only 1–10%, yet over the 3 years of observation the proportion of PBMCs that contained LASN vector increased as judged by Southern blots.

An alternate explanation for differences in vector activity is that there are inherent differences between transformed and nontransformed cells that affect retroviral vector expression. If this proves to be the case, the common use of continuously growing, transformed cell lines in the design and testing of genetic vectors may be misleading if the intended use of the vectors is transduction of normal cells.

The clinical course of this and another patient treated with activated, expanded autologous lymphocytes exposed to the retroviral ADA vector has recently been described (Blaese *et al.*, 1995). The exact contribution of retroviral ADA gene transfer relative to other components of therapy (*e.g.*, infusion of activated autologous lymphocytes and ongoing treatment with parenteral ADA enzyme) to the observed clinical improvement is difficult to assess without a randomized prospective trial. Nonetheless, the clear demonstration of vector activity in this pilot study demonstrates that therapeutic retroviral vector-mediated gene transfer in humans is technically feasible and justifies other clinical trials. The observation of long-term expression of vector genes in T lymphocytes *in vivo* suggests other applications. T lymphocytes survive for many years in immunologically normal hosts. Given their longevity and the ease with which they can be collected and manipulated *in vitro*, lymphocytes may be attractive vehicles for disorders other than immunodeficiencies in which long-term gene expression and systemic protein delivery is necessary.

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Successful Peripheral T-Lymphocyte–Directed Gene Transfer for a Patient With Severe Combined Immune Deficiency Caused by Adenosine Deaminase Deficiency

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Ten patients with adenosine deaminase deficiency (ADA$^-$) have been enrolled in gene therapy clinical trials since the first patient was treated in September 1990. We describe a Japanese ADA$^-$ severe combined immune deficiency (SCID) patient who has received periodic infusions of genetically modified autologous T lymphocytes transduced with the human ADA cDNA containing retroviral vector LASN. The percentage of peripheral blood lymphocytes carrying the transduced ADA gene has remained stable at 10% to 20% during the 12 months since the fourth infusion. ADA enzyme activity in the patient's circulating T cells, which was only marginally detected before gene transfer, increased to levels comparable to those of a heterozygous carrier individual and was associated with increased T-lymphocyte counts and improvement of the patient's immune function. The results obtained in this trial are in agreement with previously published observations and support the usefulness of T lymphocyte-directed gene transfer in the treatment of ADA$^-$SCID.

ADENOSINE DEAMINASE (EC3.5.4.4; ADA) is an enzyme in the purine salvage pathway that is critical for the deamination of adenosine and deoxyadenosine and consequent formation of inosine and deoxyinosine, respectively. The deficiency of ADA impairs the function of the human immune system resulting in severe combined immunodeficiency (SCID) characterized by severe T lymphocyte dysfunction and agammaglobulinemia.[1-3] The clinical course of inherited ADA deficiency (ADA$^-$) ranges from the rapidly fatal, early onset of classical ADA$^-$SCID to the minimally dysfunctional immune system of patients presenting "partial" ADA deficiency.[4,5] A recent review classified ADA deficiency into four types as determined by the age at clinical onset and suggested that these variants are the result of different, specific mutations resulting in various severities of enzyme dysfunction.[6]

Although the current treatment of choice for ADA$^-$SCID is an HLA-matched bone marrow transplant,[7] less than one third of patients have access to an appropriate donor. An alternative is enzyme replacement using polyethene glycol-modified bovine ADA (PEG-ADA). This represents a life saving, but costly, therapeutic option for the patients that do not have an HLA-matched donor.[8,9] Although enzyme replacement with PEG-ADA partially reconstitutes the immune function of most patients with ADA$^-$SCID, a few patients have been unresponsive to PEG-ADA.

The determination of the complete sequence of both the ADA cDNA[10-12] and the genomic ADA structural gene[13] has facilitated the molecular analysis of ADA$^-$ patients and permitted identification of various genetic mutations in unrelated ADA$^-$ patients. Early identification of the mutant gene led ADA$^-$SCID to become the first disorder to be treated by gene therapy. Two ADA$^-$SCID patients who had manifested differing levels of severity of persistent immunodeficiency despite continuous treatment with PEG-ADA thus were enrolled in 1990.[14] Since then, 10 patients with ADA$^-$SCID have undergone gene therapy as recently described.[14-17] The strategies adopted in these trials have differed and the efficacy of treatment has varied.

We report the molecular analysis of the genetic defect in an ADA$^-$SCID patient enrolled in the first gene therapy protocol in Japan and analyze the clinical results obtained during the first 18 months of this clinical trial.

MATERIALS AND METHODS

Cell culture. B-lymphoblastoid cell lines (B-LCL) were established from our ADA$^-$SCID patient, his parents and a healthy volunteer by Epstein-Bar Virus (EBV) transformation. B-LCL were maintained in RPMI-1640 medium (GIBCO-BRL, Grand Island, NY) with 10% fetal calf serum (FCS; GIBCO-BRL) and 50 mmol/L β-mercaptoethanol (Sigma Chemical Co, St Louis, MO).

Sequence analysis of patient's ADA cDNA and genomic DNA. For the analysis of the ADA cDNA sequence, total cellular RNA was isolated from B-LCL using TRIZOL Reagent (GIBCO-BRL). First-strand cDNA was synthesized from 2 μg of total cellular RNA (First strand synthesis kit; Promega, Madison, WI). Full-length ADA cDNA fragments extending from the translation start site codon to 230 base pair (bp) 3′ of the stop codon were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR). Oligonucleotide primers for RT-PCR were as follows: sense primer; CCATGGCCCAGACGCCC-GCCTT, antisense primer; ACCATAGCCCATGTGCAAGGGC. Reactions containing 0.5 μL (2.5 U) *Taq* polymerase (*TaKaRa Ex Taq*, TaKaRa Shuzo Co, Ltd, Tokyo, Japan) were incubated for 30 cycles of 60 seconds at 92°C, 90 seconds at 58°C, and 180 seconds at 72°C with the extension time at 72°C increased to 10 minutes in the last cycle. Amplified products were isolated from 1.0% agarose gel and then subcloned into pCR II vector (Invitrogen, San Diego, CA). Sequence analysis of double-stranded DNA was performed using Sequenase version II DNA sequencing kit (Amersham Life Science, Arlington

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Heights, IL) with [^{35}S]dATP (Amersham Life Science) and a series of ADA-specific primers. Amplified products were sequenced through a 6% acrylamide gel (National Diagnostics, Atlanta, GA). To analyze the ADA genomic sequence, high molecular DNA was obtained from B-LCL by standard techniques.[18] Primers and PCR conditions for amplification of ADA all exons have also been described previously.[19-21] Amplified products were isolated from agarose gel and sequenced directly using the Thermal Cycler DNA sequencing kit (Circum Vent; New England Biolabs Inc, Beverly, MA). ADA cDNA sequences are numbered relative to the start site of translation and genomic DNA according to Wiginton et al.[13]

Southern blot analysis. High molecular weight DNA from B-LCL was digested with restriction endonuclease *Rsa* I, separated in 1.0% agarose gel, and transferred onto a nylon membrane (Biotrace HP; Gelman Sciences, Ann Arbor, MI). Filters were then hybridized to a ^{32}P randomly labeled 444-bp *Rsa* I-*Pst* I fragment from the ADA cDNA.

Retroviral-mediated gene transfer into patient's peripheral T cells. The clinical protocol used here has been described elsewhere.[22] Briefly, peripheral T lymphocytes from the patient were obtained by apheresis (CS3000 plus, Baxter Corp, Chicago, IL), isolated by density gradient centrifugation, and then maintained in AIM-V medium (GIBCO-BRL) supplemented with 5% FCS (GIBCO-BRL), 100 U/mL of recombinant human IL-2 (rIL-2, SHIONOGI, Osaka, Japan) and 10 ng/mL of anti-CD3 antibody (Orthoclone OKT3 Injection; Ortho, Raritan, NJ) in gas-permeable culture bags (Nipro Pretobag; Nishyo, Osaka, Japan). After 72 hours, half of the medium was removed and replaced with supernatant containing the LASN retroviral vector[23] supplemented with interleukin-2 (IL-2) and 10 μg/mL of protamine (Shimizu, Shimizu City, Japan). The LASN supernatant, prepared under Good Manufacturing Practices guidelines, was supplied by Genetic Therapy Inc (Gaithersburg, MD). The transduction procedure was repeated twice following an optimized transduction protocol combining low-temperature (32°C) incubation and centrifugation.[24] After two rounds of transduction, the virus supernatant was replaced with fresh medium supplemented with IL-2 and the cells were cultured for an additional 6 days. At the 11th day of culture, the cells were harvested and washed extensively with saline containing 0.5% human albumin and then reinfused into the patient.

Analysis of the inserted proviral genome by semi-quantitative PCR. Sense (GAGGCTGTGAAGAGCGGCAT) and anti-sense (CTCGAAGTGCATGTTTTCCT) primers were designed to match the sequence of the start site of exon 7 and the end of exon 8, respectively. Using these primers, the amplification of DNA samples from vector-containing cells generates two bands; the larger one (250 bp) derived from the endogenous ADA gene containing intron 7 (76 bp) and the smaller one (174 bp) from the LASN provirus. To evaluate the frequency of transduced cells in the patient's peripheral blood, a standard curve was prepared from a serial dilution of in vitro-transduced and G418-selected B-LCL with untransduced cells. The ratio of the amount of amplified ADA cDNA derived from the integrated vector and the amplified genomic sequence was calculated after hybridization with an ADA cDNA probe.

Thin-layer chromatography (TLC) analysis of ADA enzyme activity. Mononuclear cells were washed twice with phosphate-buffed saline to remove FCS and then suspended in 100 mmol/L Tris, pH 7.4 containing 1% bovine serum albumin. Cell lysates were obtained by 5 rapid freeze-thaw cycles. Cellular debris was removed by centrifugation and the lysates were stored at −80°C until used. ADA enzyme activity was assayed by the measurement of the conversion of [^{14}C] adenosine (Amersham Life Science) to [^{14}C] inosine and [^{14}C] hypoxanthine followed by TLC separation of the reaction products performed as previously described.[25] The results were expressed as nanomoles of inosine and hypoxanthine produced per min by 10^8 cells (nmol/min/10^8 cells).

RESULTS

Clinical course. The patient is a 5-year-old Japanese male. Symptoms including a chronic productive cough and a purulent nasal discharge began at 8 months of age. At 10 months he developed respiratory distress and was hospitalized for the treatment of severe pneumonia that was unresponsive to antibiotics. On admission at age 10 months, the patient had lymphopenia (absolute lymphocyte count 520/μL), with few mature T and B lymphocytes (CD3, 125/μL; CD4, 62/μL; CD8, 41/μL; CD19, 26/μL) and low serum Ig levels (IgG, 342 mg/dL; IgA, 18 mg/dL; and IgM, 60 mg/dL). Both humoral and cellular immunity were defective, with undetectable isohemagglutinins and absent T-cell proliferative responses to phytohemagglutinin, Concanavalin A, and pokeweed mitogen. Since ADA activity in his red blood cells (RBCs) was undetectable and the deoxyadenosine triphosphate (dATP) level was 506 nmol/mL RBCs (normal <2 nmol/mL), the diagnosis of SCID due to ADA deficiency of the "delayed onset" type[6] was established. In the absence of a suitable bone marrow donor, PEG-ADA therapy was initiated at 15 months of age and supplemented with intravenous Ig (IVIG). After treatment with PEG-ADA (37.5 U/kg/wk), the plasma ADA activity in the patient's peripheral blood increased from 0.14 to 53.15 μmol/h/mL and the peripheral blood lymphocyte (PBL) count increased to the range of 1,000 to 2,000/μL. Despite continuous PEG-ADA treatment, however, his Ig levels remained below normal and the lymphopenia recurred during the second year of enzyme replacement. The PBL count decreased to less than 1,000/μL with CD3$^+$ cell counts of 400/μL before the start of gene therapy (PBL, 702/μL; CD3, 400/μL; CD4, 205/μL; CD8, 191/μL; CD19, 57/μL on protocol day 0).

Identification of mutations responsible for ADA deficiency. To analyze mutations in our patient, we amplified full-length ADA cDNA from the patient's EBV transformed B-LCL by RT-PCR. Sequence analysis revealed that all of the clones (6/6) carried a G^{632} to A transition resulting in replacement of the arginine residue by histidine at codon 211 (Fig 1A). The mutation eliminates a recognition site for the restriction enzyme *Rsa* I. We took advantage of this feature to distinguish the mutated allele from the normal allele.[19] High molecular weight DNA extracted from the patient's B-LCL was digested with *Rsa* I, blotted and hybridized to an ADA cDNA probe spanning the region from this mutation site in exon 7 to the end of exon 11 (Fig 1B). *Rsa* I digestion showed both a normal (3.1 kb) and a larger fragment (4.4 kb) in the patient lane, indicating that the patient was heterozygous for loss of the *Rsa* I recognition site in exon 7. To determine the parental derivation, amplified genomic fragments spanning intron 6 to intron 9 of the patient and his parents were digested with *Rsa* I and electrophoresed in 2% agarose gel (Fig 1C). The patient's digestion pattern was identical to that obtained from the analysis of the father's DNA, indicating that this mutation was derived from the paternal allele.

Northern blot analyses showed that the quantity of the ADA message from both the patient and his mother was reduced to approximately half of control (data not shown). All cDNA clones carried the paternal missense mutation, suggesting that the mutation derived from the maternal allele resulted in undetectable mRNA. To characterize this mutation, we ana-

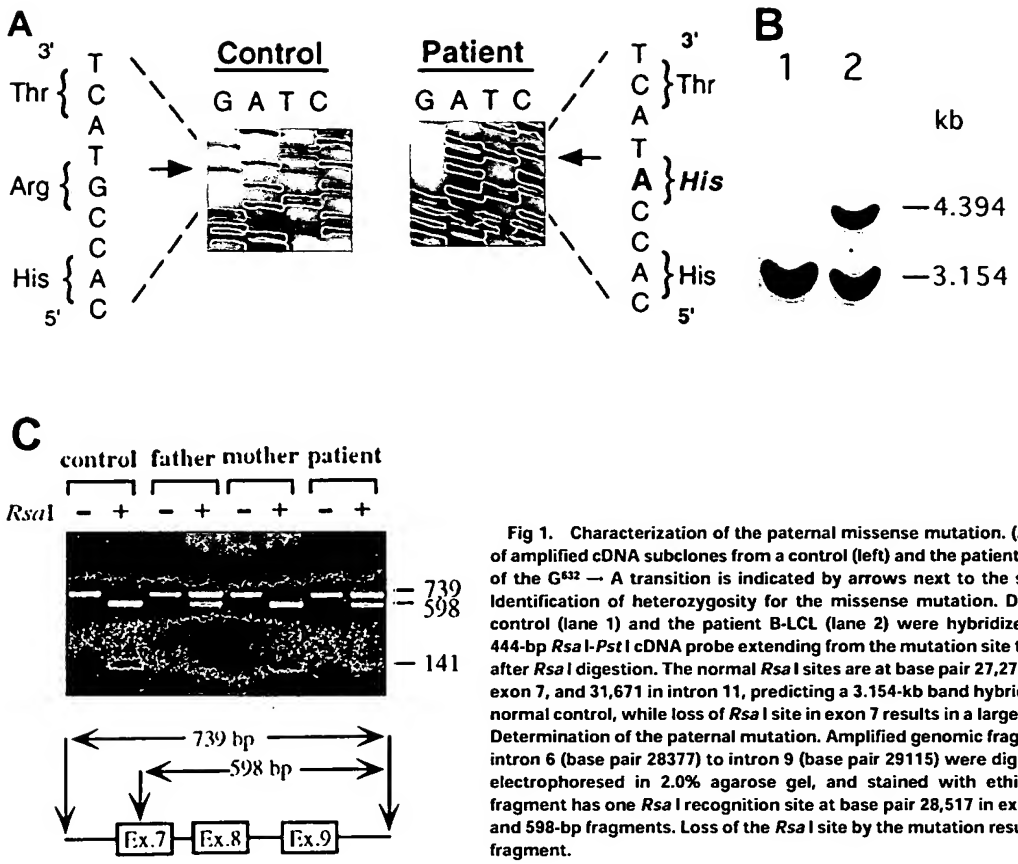

Fig 1. Characterization of the paternal missense mutation. (A) Sequence (sense) of amplified cDNA subclones from a control (left) and the patient (right). The position of the $G^{632} \rightarrow A$ transition is indicated by arrows next to the sequence ladder. (B) Identification of heterozygosity for the missense mutation. DNA samples from a control (lane 1) and the patient B-LCL (lane 2) were hybridized to a radiolabeled 444-bp *Rsa* I-*Pst* I cDNA probe extending from the mutation site to the end of exon 11 after *Rsa* I digestion. The normal *Rsa* I sites are at base pair 27,276 in exon 6, 28,516 in exon 7, and 31,671 in intron 11, predicting a 3.154-kb band hybridized to the probe in normal control, while loss of *Rsa* I site in exon 7 results in a larger band (4.394 kb). (C) Determination of the paternal mutation. Amplified genomic fragments (739 bp) from intron 6 (base pair 28377) to intron 9 (base pair 29115) were digested with *Rsa* I and electrophoresed in 2.0% agarose gel, and stained with ethidium bromide. The fragment has one *Rsa* I recognition site at base pair 28,517 in exon 7, predicting 141- and 598-bp fragments. Loss of the *Rsa* I site by the mutation results in an undigested fragment.

lyzed exons 1 to 11 by PCR amplification of genomic DNA and direct sequencing. Sequence analyses of the amplified fragments including exon 2 showed the patient to be heteroallelic for a splice site mutation at the first position of intron 2 ($G^{+1} \rightarrow A$ transversion) (Fig 2A). This mutation eliminates a recognition site for the restriction enzyme *Bsp*MI. *Bsp*MI digestion showed that the patient and his mother were heterozygous for this mutation, while the father showed a normal individual digestion pattern (Fig 2B). Reports of mutation analyses of other patients have shown that a mutation affecting a mRNA splicing mechanism may give rise to a nonfunctional or unstable mRNA.[26,27] This mechanism is also supported by the fact that *Rsa* I digestion showed that all full-length cDNA clones (48/48) from the patient's B-LCL carried the paternal G^{632} to A missense mutation.

Retroviral mediated gene transfer into peripheral T cells. At the age of 4, the patient was enrolled in a clinical gene therapy trial that repeated the protocol of the first gene therapy experiment at the National Institutes of Health (NIH) in 1990.[22] The patient's peripheral mononuclear cells, obtained by apheresis, were stimulated with IL-2 (100 U/mL) and anti-CD3 antibody (OKT3; 10 ng/mL). After 72 hours of stimulation, they were transduced twice during the next 48 hours by exposure to the ADA retroviral vector LASN, expanded 20- to 50-fold in number by culturing for 6 days after the beginning of transduction, and then reinfused into the patient (see Materials and Methods). No selection procedure to enrich for gene-transduced cells was performed. Semiquantitative PCR of the cells in the first and second infusions revealed that the frequency of the vector-carrying cells ranged from 3% to 7% (data not shown).

Clinical course after gene therapy. The patient received a total of 10 infusions over the 18-month period (Fig 3). A striking increase in lymphocyte number was observed early in the trial, followed by a gradual return to the basal level. This was followed by a sustained increase after the 8th infusion (protocol day 322) and the patient's PBL count has since remained in the normal range (PBL, 1,980/μL; CD3, 1,822/μL; CD4, 240/μL; CD8, 1,538/μL; CD19, 154/μL on protocol day 429). Progressive inversion of CD4/CD8 ratio has been observed since the 4th infusion due to an increase of the absolute $CD8^+$ cell count. This phenomenon is thought to be the result of preferential proliferation of $CD8^+$ cells during in vitro culture and transduction. ADA enzyme activity, nearly undetectable in the patient's lymphocytes before gene therapy, also increased progressively after the 7th infusion (protocol day 252) and reached 27 U on protocol day 476, which is approximately comparable to that of a heterozygous carrier individual (the patient's mother, 34.8 U).

The number of transduced cells in the patient's peripheral blood were assessed by semiquantitative PCR using PBL obtained before each infusion (Fig 4). The frequency of the genetically modified cells increased with the number of infusions of the ADA gene transduced lymphocytes and exceeded 10% of total circulating mononuclear cells just before the 5th infusion (on protocol day 126; Fig 4, lane 4). The frequency

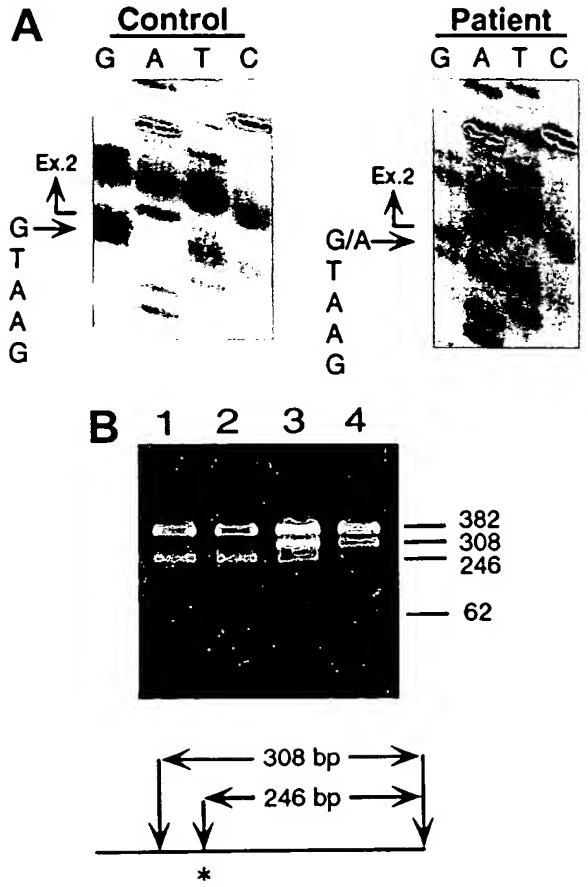

Fig 2. Identification of the maternal mutation at the splice donor site in intron 2. (A) Sequence (sense) of the exon 2/intron 2 junction in amplified genomic DNA. Genomic fragments containing exon 2 were amplified from a control (left) and the patient (right) and sequenced directly. A mutation at the splice donor site in intron 2 ($G^{+1} \rightarrow A$) is indicated by arrows. (B) Detection of the splice site mutation by the *Bsp*MI digestion. Amplified genomic fragments (690 bp) from intron 1 (bp 14,901) to intron 2 (base pair 15,590) was digested with *Bsp*MI, electrophoresed in 2.0% agarose gel, and stained with ethidium bromide. The fragment has two *Bsp*MI recognition sites at bp 15,282 and 15,344, predicting 62-, 246-, and 382-bp fragments in the control lane. Loss of the *Bsp*MI site (base pair 15,282) by the mutation results in the undigested fragment (308 bp). Lane 1, control; lane 2, father; lane 3, mother; and lane 4, patient. *Bsp*MI digestion shows the patient and his mother were heterozygous for the splice site mutation.

measured before each of the 6th through 10th infusions (on protocol days 210 to 462) has remained stable at 10% to 20%.

To evaluate the functional consequences of the ADA enzyme activity that had been induced by gene transfer, we compared the patient's immune function before and after the treatment (Table 1). Eleven months after beginning gene therapy, the patient's isohemagglutinin titer (IgG) increased from undetectable to 1:16 and delayed-type hypersensitivity (DTH) skin test responses became stronger. The interval between IVIG infusions which were given monthly before gene therapy, was widened and eventually stopped after gene therapy. Despite this, the patient's serum Ig levels gradually increased and have remained normal for more than a half year without additional IVIG treatment (Fig 3 and Table 1). These results suggest that the accumulated genetically corrected T lymphocytes in the patient's peripheral blood are associated with improvement of cellular and humoral immune responses and an increase in his circulating lymphocyte count. Although he sometimes became transiently febrile after infusions, the patient showed no serious adverse reactions to the treatments.

DISCUSSION

Advances in molecular biology during the past 3 decades have suggested that gene transfer could provide a new approach to the treatment of inherited diseases as well as acquired disorders such as cancer and acquired immune deficiency syndrome.[28] The number of active gene therapy protocols has increased greatly since the first clinical gene therapy trial.[29] ADA⁻SCID is one of the few early candidate disorders suitable for such interventions.[30] Accordingly, 10 ADA⁻SCID patients have been enrolled in gene therapy clinical protocols that employed different strategies, retroviral vector designs, and target cell populations. The results obtained from these trials have recently been reported.[14-17]

This trial of gene therapy for an ADA⁻SCID patient in Japan began in August 1995. Over the next 18 months he received a total of 10 infusions of cultured-expanded autologous T cells that had been transduced with the LASN retroviral vector. After an initial period of fluctuating counts, the patient's T cells stabilized in the normal range and this has been sustained for the last half year. The frequency of integrated provirus in the patient's peripheral blood increased to approximately 15% (0.1 to 0.2 proviral copies/cell) by the 4th infusion and has remained stable since that time. The patient's cell associated adenosine deaminase enzyme activity has increased from barely detectable before treatment to values approaching those found in the peripheral mononuclear cells of his heterozygous carrier mother. Delayed hypersensitivity skin tests, a measure of T-cell function, have improved. Isohemagglutinin titers have also increased and his dependence on infusions of normal gammaglobulin has eased. The patient has gained 3 kg in weight during this trial. He is still receiving periodic PEG-ADA replacement and is attending public school with no more infections than his classmates.

The period of observation has simply not been sufficient to assess the full breadth or the duration of this improved clinical status and immune responsiveness. Further, additional studies will be required to reconcile the apparent dissociation between the level of T-cell ADA observed and the proportion of cells containing integrated vector at different time points. Also, the effect of withdrawal of the exogenous PEG-ADA treatment must await more complete characterization of the quality of the patient's immune system and the repertoire of specificities represented in the transduced T-cell population.

Four gene therapy clinical trials including 10 ADA⁻SCID patients have been performed since the first trial in 1990. Although these trials provided much data that suggested how future gene therapy might be improved by changing retroviral vector design, transduction methods and target cell populations, we found it difficult to compare the efficacy of these various trials because of differences inherent within these basic strate-

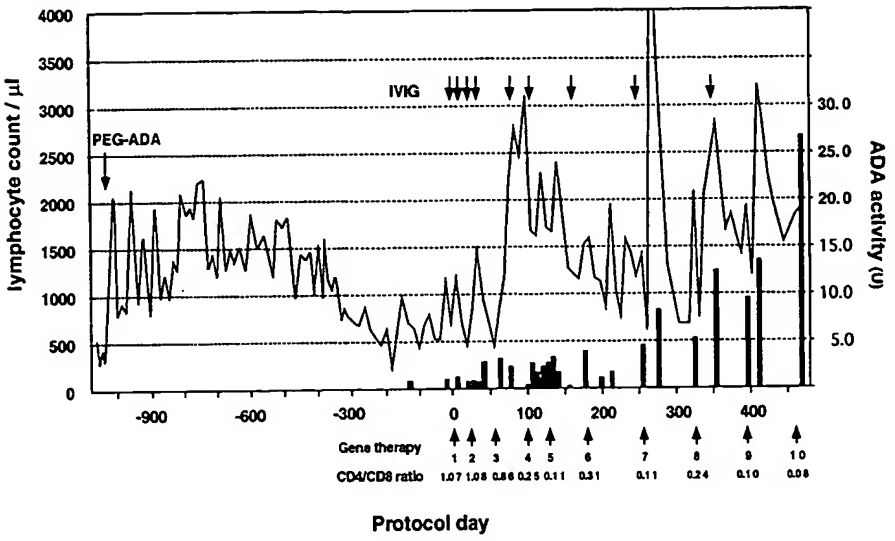

Fig 3. Clinical course before and after gene therapy. Gene therapy started on August 1, 1995 (protocol day 0) with the patient receiving a total of 10 infusions to date. PEG-ADA therapy was initiated at 15 months of age. The lymphocyte count is indicated by a solid line and CD4/CD8 ratio was measured using PBL before infusion. ADA activity shown by a solid bar is expressed as nanomoles of inosine and hypoxanthine produced per minute by 10^8 cells. Replacement of IVIG after gene therapy is shown as an arrow. The patient received a Ig replacement (2.5 g) monthly before gene therapy.

gies. Our trial has been performed following the identical protocol and vector preparations and autologous T lymphocyte isolation procedures that were used in the NIH trial. From this perspective, our trial provides an additional opportunity to evaluate the effectiveness of peripheral T lymphocyte-directed gene therapy for ADA$^-$SCID patients. Interestingly, the clinical course of our patient is quite similar to that observed in patient 1 in the NIH trial. Both trials have shown high gene transfer efficiency, remarkable increase of the ADA enzyme activity and eventual improvement of immune function. In contrast, patient 2 in the NIH trial experienced a low gene transfer efficiency and no significant increase in the ADA enzyme activity even though she exhibited some increase in immunological function. Although the factors leading to this difference have not yet been completely identified, a striking difference in the transduction efficiency of peripheral T cells between the three patients may be relevant. Transduction efficiencies before infusion were 3% to 7% for the present case, 1% to 10% for patient 1 and 0.1% to 1% for patient 2 in the NIH trial. An abbreviated proliferative capacity of patient 2 in the NIH trial was also observed. In addition, a contribution of the development of an immune response to the neomycin resistance gene must be considered since the existence of dominant selectable markers of nonhuman origin may result in unwanted immune reactivity that could eliminate or functionally impair transgene-expressing cells.[31]

The severity of the underlying ADA gene defects could also affect gene transfer. In addition to the mutation analysis reported here, specific ADA gene defects have also been reported for the two NIH patients.[20] These three cases can be classified by the severity of their clinical presentation. Both the present case and patient 1 in the NIH trial are of the "delayed onset" type, have splice site mutation defects and have achieved significant levels of "gene-corrected" circulating cells. However, the NIH patient 2 carries compound missense mutations and has manifested low transduction efficiency despite her less severe "late onset" type of presentation at age 5. Although there are insufficient numbers of treated patients to draw firm conclusions at this point, it does appear thus far that the responses of patients with "more severe" gene defects and clinical presentations are at least as responsive as cases with "milder ADA defects."

It should be noted that the ADA gene transduced T lymphocytes possess a selective advantage over the nontransduced cells due to the latter's high intracellular concentration of deoxyadenosine.[32,33] In the ADA$^-$ newborn trial using gene-corrected CD34$^+$ cells obtained from the patient's umbilical cord blood,[16] LASN vector was detected in the peripheral blood T cells of these patients at a stable frequency of approximately 0.01% during the first 18 months of observation. Then, after a 50% reduction in their weekly dose of PEG-ADA, the proportion of

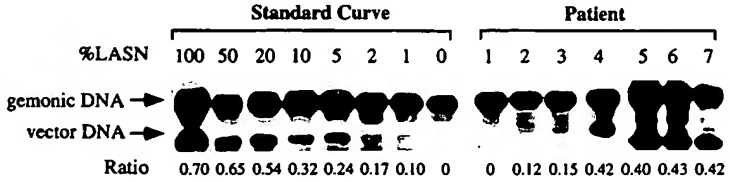

Fig 4. Semiquantitative PCR analysis to evaluate the frequency of vector-carrying cells in the patient's peripheral blood. Patient's mononuclear cells were obtained before the indicated infusion: before gene therapy (lane 1), 2nd infusion (protocol day [D] 21-lane 2), 4th infusion (D 98-lane 3), 5th infusion (D 126-lane 4), 6th infusion (D 175-lane 5), 8th infusion (D 322-lane 6, and 10th infusion (D 462-lane 7), and assayed for the frequency of vector containing cells by semiquantitative PCR. A standard was prepared by diluting cells containing the LASN vector with nontransduced cells. The ratio was determined by comparing the density of the cDNA derived band to that of the genomic DNA derived band.

Table 1. Isohemagglutinin Titer, DTH Skin Test Reactivity, and Ig Levels Before and After Gene Therapy

		Before	After
Isohemagglutinin titer	(IgG)	<2	16
DTH skin test (mm)	PPD*	8 × 7	12 × 10
	Candida	3 × 3	18 × 9
	Tetanus	N.D.	6 × 5
Igs	IgG	720	811
	IgA	20	53
	IgM	84	128

Isohemagglutinin titer and DTH skin tests were tested using standard protocols before gene therapy (before) and at 11 months after the beginning of gene therapy (after) while on PEG-ADA. The patient serum Ig levels were measured just before the Ig replacement on protocol day −60 (before) and 478 (after). The patient received the last Ig replacement at protocol day 348.

*The patient had been immunized with BCG at 5 months of age.

ADA vector-containing T cells in the blood increased to approximately 10% in each case (D.B. Kohn, personal communication, September 1995). In the present case, the dosage schedule of PEG-ADA enzyme has remained constant since the beginning of the trial (18 U/kg/wk on the protocol day 431), during which time the patient's immune function has substantially improved. It might be expected that the proportion of the transduced cells in the patient's PBL will increase as the PEG-ADA dosage is decreased.

To date, three clinical trials have been performed to assess the possibility of treating ADA⁻SCID patients by correcting hematopoietic progenitor cells.[15-17] The results obtained from these trials suggest that cord blood provides a stem cell population more suitable for efficient retroviral-mediated gene transfer than does bone marrow. Taken with the observations made in the NIH trial, our results strongly suggest that the effectiveness of T lymphocyte-directed gene transfer is a viable addition to the treatment programs that should be considered for ADA⁻SCID patients. After additional courses of treatment and continued observation to determine the breadth and durability of these positive responses, we hope to reduce or eliminate exogenous ADA enzyme supplementation in this patient. Improvements in vector design to permit higher levels of ADA expression and innovative strategies that provide greater efficiency of stem cell gene transduction may make gene therapy the treatment of choice for ADA⁻SCID patients.

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